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DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

WILD BOAR AS A SOURCE OF SELECTED EMERGING AND RE-EMERGING PATHOGENS: APPLICATION OF MICROARRAY TECHNOLOGIES

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To my family

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Chapter 1- Introduction

The introduction of the present thesis starts with a review of the current knowledge on the most important viral, bacterial and parasitic infections of wild boar, with a focus on their diagnosis, followed by a description of novel diagnostic technologies, namely microarrays and multiplex bead assay.

Chapter 2 - A serosurvey for selected pathogens in Greek European wild boar

The aim of this study was to investigate the seroprevalence rate for 10 selected pathogens, important for livestock and/or public health, in wild boar from different areas of Greece and to correlate results with environmental factors using a geographical information system.

Serum samples, collected from 94 European wild boar (*Sus scrofa*) during the hunting seasons 2006-2010, were examined. The assays used were commercial indirect enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against porcine circovirus-2 (PCV-2), porcine reproductive and respiratory syndrome (PRRS) virus, Aujeszky's disease virus, influenza A virus, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Salmonella* spp, *Trichinella* spp and indirect immunofluorescence antibody (IFA) test for the detection of antibodies against *Toxoplasma gondii* and *Neospora caninum*. The sample collection area was located in the field using handheld Global Positioning System (GPS) units or longitude and latitude information. GIS layers were created to represent the geographic locations of the sampling and of the nearest free-ranging swine farms. Cluster analysis for seropositivity to at least one of the pathogens was performed. Also, the relationships between seropositivity to each pathogen and wild boar sex, selected environmental variables (altitude, distance from the nearest free-ranging swine farms, land use, land cover) and density of wild boar population was examined.

Antibodies against PCV-2, PRRS virus, Aujeszky disease virus, influenza A virus, *A. pleuropneumoniae*, *M. hyopneumoniae*, *Salmonella* spp, *Trichinella* spp, *T. gondii* and *N. caninum* were detected in 19.1%, 12.8%, 35.1%, 1.1%, 57.4%, 0%, 4.3%, 6.4%, 5.2% and 1.1% of the samples, respectively. Cluster analysis revealed a hot spot of seropositivity near Bulgarian border. Seropositivity to Aujeszky's disease virus was more common among female animals. There were no significant associations between environmental factors (altitude, distance from the nearest free-ranging swine farms, land use, land cover) or density of wild boar population and seropositivity with the only exception of PRRS virus where a borderline association with land cover ($p=0.05$) was found (higher seopositivity in cultivated and managed areas).

These results indicate exposure of wild boar to most of these pathogens, raising concern about the possibility that this species may pose a significant health risk for livestock and/or humans.

Chapter 3 - Development of a multiplex bead assay for the detection of serological responses to *Brucella* species that overcomes the cross-reactivity with *Yersinia enterocolitica* O:9 in domestic pig and wild boar population

The aim of this study was to develop a multiplex bead assay using a rough *Brucella* lipopolysaccharide (rLPS) antigen, a whole cell *Brucella suis* 1330 smooth antigen, and a whole cell *Yersinia enterocolitica* O:9 antigen, that not only discriminates *Brucella* seropositive from *Brucella* seronegative domestic pigs and wild boar but also overcomes the cross reactivity with *Y. enterocolitica* O:9.

One hundred twenty six domestic pig sera allocated into three groups were used: Group A (*Brucella* infected) containing 29 sera collected from pigs confirmed by culture to be infected with *B. suis* biovar 2 (25/29) or biovar 1 (4/29). Group B (non *Brucella* infected) containing 80 randomly selected sera collected from herds within Great Britain. Great Britain is officially brucellosis-free. Group C containing 17 sera from Great Britain confirmed as false positive serological reactors (FPSR) during routine testing. Additional 49 Eurasian wild boar sera allocated into two groups: group

A containing 18 positive (*Brucella* seropositive) and group B containing 31 negative (*Brucella* seronegative) sera were included in the study.

Twenty six of the 29 *Brucella* infected domestic pigs (23/25 *Brucella* infected by biovar 2 and 3/4 infected by biovar 1) were shown to be positive using the rLPS antigen, while all (17/17) FPSR domestic pigs and 75/ 80 non *Brucella* infected domestic pigs were negative. The same antigen detected 15/18 of the seropositive and it was negative in all (31/31) of the confirmed seronegative wild boar sera. The smooth *B. suis* 1330 antigen detected all (29/29) *Brucella* infected domestic pigs, was negative in all (80/80) non *Brucella* infected, however, it showed positive reactions in in most (13/17) FPSR. In wild boar the same antigen detected all (18/18) seropositive and it was negative in 30/31 seronegative animals. Finally, the ratio of the smooth *B. suis 1330* and the smooth *Y. enterocolitica* O:9 normalized median fluorescence intensity (MFI) values discriminated with 100% sensitivity and 100% specificity *Brucella* infected from the FPSR domestic pigs.

These results demonstrate the potential of the multiplex bead assay for use in surveillance of brucellosis in pigs and wild boar and furthermore, it has a potential to overcome the cross-reactivity with *Y. enterocolitica*.

Chapter 4 - Diagnostic performance of a multiplex bead assay and a serology microarray technology as serodiagnostic tools for the simultaneously detection of antibodies against *Mycobacterium bovis*, *Brucella suis* and *Trichinella spiralis* in wild boar

The aim of this study was to evaluate the diagnostic performance of two multiplexed assays, a multiplex bead assay and a serology microarray technology, for the simultaneous detection of antibodies against *Mycobacterium bovis*, *Brucella suis* and *Trichinella spiralis* and to compare it with that of conventional single-pathogen indirect "in house" ELISAs for bovine tuberculosis and porcine brucellosis and with a commercial ELISA for wild boar trichinellosis.

One hundred sixty nine sera from Eurasian wild boar of known TB serology status (64 seropositive, 105 seronegative), 68 sera of known *Brucella* serology status (29 seropositive, 39 seronegative) and 118 sera of known trichinelosis status (21 seropositive, 97 seronegative) were usedfor the development and the evaluation of the multiplex bead assay and for the evaluation of the serology microarray.

A recombinant MPB83 antigen, a whole cell *B. suis* 1330 antigen and an E/S *T. spiralis* antigen were used for the development and the evaluation of the multiplex bead assay. A developed microarray chip containing 116 different antigens of 63 different viral, bacterial and parasitic pathogens, including the above antigens, was evaluated. Due to the lack of commercial availability of tests for detection of antibodies against tuberculosis and brucellosis in wild boar, "in house" ELISAs were performed using the recombinant MPB83 antigen and the whole cell *B. suis* 1330 antigen. The results for each antigen (MPB83, *B. suis* 1330 and E/S *T. spiralis*) were compared among the serological assays (multiplex bead assay, serology microarray technology, "in house" ELISA) as well as between each serological assay and the gold standard.

The multiplex bead assay discriminated TB seropositive from seronegative wild boar with sensitivity (Se) of 98.4% and specificity (Sp) of 85.7%, whereas the corresponding values for brucellosis were 100% and 97.4%, respectively and for trichinellosis 90.5% and 99%, respectively. Comparable results were obtained with the serology microarray technology (Se and Sp of 92% and 92.4% for TB, of 100% and 91.7% for *B. suis* and of 75% and 98.7% for *T. spiralis*). Most of the results for the multiplexed serological assays agreed with the gold standard ELISA results, with exception of the multiplex bead assay's results for TB.

Chapter 5 – Final comments and future perspectives

In this chapter the reasons that wild boar should be included in surveillance and monitoring programmes at national level and the potential of microarray technology and multiplex bead assay as serological tools are summarized.

ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ

ΕΡΓΑΣΤΗΡΙΟ ΜΙΚΡΟΒΙΟΛΟΓΙΑΣ ΚΑΙ ΠΑΡΑΣΙΤΟΛΟΓΙΑΣ

Ο ΡΟΛΟΣ ΤΩΝ ΑΓΡΙΟΧΟΙΡΩΝ ΩΣ ΠΗΓΗ ΜΕΤΑΔΟΣΗΣ ΑΝΑΔΥΟΜΕΝΩΝ ΚΑΙ ΕΠΑΝΑΔΥΟΜΕΝΩΝ ΛΟΙΜΩΔΩΝ ΝΟΣΗΜΑΤΩΝ: ΕΦΑΡΜΟΓΗ ΤΕΧΝΟΛΟΓΙΑΣ ΜΙΚΡΟΣΥΣΤΟΙΧΙΩΝ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΑΝΤΩΝΙΑ ΤΟΥΛΟΥΔΗ

Κτηνίατρος

ΚΑΡΔΙΤΣΑ 2015

στην οικογένειά μου

ΤΡΙΜΕΛΗΣ ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ

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ΠΕΡΙΛΗΨΗ

Κεφάλαιο 1 – Εισαγωγή

Η εισαγωγή της παρούσας διδακτορικής διατριβής περιλαμβάνει την ανασκόπηση των σύγχρονων δεδομένων για σημαντικά ιογενή, βακτηριακά και παρασιτικά νοσήματα των αγριόχοιρων, με έμφαση στη διάγνωσή τους, καθώς και την περιγραφή νέων διαγνωστικών τεχνολογιών και συγκεκριμένα των μικροσυστοιχιών και της πολυσύνθετης δοκιμής μαγνητικών σφαιριδίων.

Κεφάλαιο 2 – Μία ορολογική μελέτη για επιλεγμένα παθογόνα στους ελληνικούς αγριόχοιρους

Ο σκοπός της μελέτης αυτής ήταν να διερευνηθεί η συχνότητα της οροθετικότητας των αγριογούρουνων από διάφορες περιοχές της Ελλάδας έναντι 10 επιλεγμένων παθογόνων, σημαντικών για τα οικόσιτα ζώα ή τον άνθρωπο και να συσχετιστούν τα αποτελέσματα με περιβαλλοντικούς παράγοντες, που παρέχονται μέσω συστήματος γεωγραφικών πληροφοριών (ΣΓΠ).

Εξετάστηκαν δείγματα ορών, που συλλέχθηκαν από 94 αγριόχοιρους κατά τη διάρκεια των κυνηγετικών περιόδων 2006-2010. Οι ορολογικές εξετάσεις που χρησιμοποιήθηκαν ήταν εμπορικά διαθέσιμες έμμεσες ανοσοενζυματικές δοκιμές ανοσοπροσρόφησης (ELISA) για την ανίχνευση αντισωμάτων έναντι του κυκλοϊού-2 του χοίρου, του ιού του αναπαραγωγικού και αναπνευστικού συνδρόμου του χοίρου (ΑΑΣΧ), του ιού της νόσου του Aujeszky, της γρίπης τύπου Α των χοίρων, των βακτηρίων *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Salmonella* spp και του παρασίτου *Trichinella* spp και ο έμμεσος ανοσοφθορισμός (IFAT) για την ανίχνευση αντισωμάτων έναντι των παρασίτων *Toxoplasma gondii* and *Neospora caninum*. Οι περιοχές απ' όπου συλλέχθηκαν τα δείγματα, τοποθετήθηκαν στο πεδίο με τη χρήση του παγκοσμίου συστήματος εντοπισμού θέσης ή με τη χρήση πληροφοριών γεωγραφικού μήκους και πλάτους. Ένα ψηφιακό επίπεδο πληροφοριών δημιουργήθηκε ώστε να αντιπροσωπεύει τις γεωγραφικές θέσεις συλλογής των

δειγμάτων καθώς και τη θέση των πλησιέστερων αγροκτημάτων ημιεκτατικής εκτροφής χοίρων. Πραγματοποιήθηκε ανάλυση της διασποράς της οροθετικότητας για τουλάχιστον ένα από τα εξεταζόμενα παθογόνα. Επίσης διερευνήθηκε τυχόν συσχετισμός μεταξύ της οροθετικότητας για κάθε παθογόνο ξεχωριστά με το φύλο των αγριόχοιρων, διάφορους περιβαλλοντικούς παράγοντες (υψόμετρο, απόσταση από το κοντινότερο αγρόκτημα ημιεκτατικής εκτροφής χοίρων, χρήση και κάλυψη της γης) καθώς και με την πυκνότητα του πληθυσμού των αγριόχοιρων.

Αντισώματα έναντι του κυκλοϊού-2 του χοίρου, του ιού του ΑΑΣΧ, του ιού της νόσου του Aujeszky, των ιών γρίπης τύπου Α των χοίρων, των *A. pleuropneumoniae*, *M. hyopneumoniae*, *Salmonella* spp, *Trichinella* spp, *T. gondii* και *N. caninum* ανιχνεύθηκαν σε ποσοστό 19,1%, 12,8%, 35,1%, 1,1%, 57,4%, 0%, 4,3%, 6,4%, 5,2% και 1,1% των δειγμάτων, αντίστοιχα. Η ανάλυση διασποράς κατέδειξε ως περιοχές έντονης οροθετικότητας, τις περιοχές που συνορεύουν με τη Βουλγαρία. Η οροθετικότητα έναντι του ιού της νόσου του Aujeszky ήταν μεγαλύτερη στους θηλυκούς αγριόχοιρους ενώ δε βρέθηκε συσχέτιση μεταξύ της οροθετικότητας και των περιβαλλοντικών παραγόντων ή της πυκνότητας του πληθυσμού των αγριόχοιρων, με μόνη εξαίρεση το ΑΑΣΧ που φαίνεται να συσχετίζεται οριακά (p=0.05) με την κάλυψη γης (υψηλότερη οροθετικότητα σε καλλιεργήσιμες και διαχειριζόμενες περιοχές).

Τα αποτελέσματα αυτά καταδεικνύουν έκθεση των αγριόχοιρων στα περισσότερα από τα παραπάνω παθογόνα, γεγονός που δημιουργεί υπόνοια πως το ζωικό αυτό είδος μπορεί να αποτελεί σημαντικό κίνδυνο για τα οικόσιτα ζώα ή τη Δημόσια Υγεία.

Κεφάλαιο 3 – Ανάπτυξη μιας πολλαπλής τεχνικής μαγνητικών σφαιριδίων (multiplex bead assay) για την ανίχνευση ορολογικών αντιδράσεων έναντι των ειδών *Brucella*, παρεμποδίζοντας τη διασταυρούμενη αντίδραση με την *Yersinia enterocolitica* O:9 σε οικόσιτους χοίρους και αγριόχοιρους.

Ο σκοπός της μελέτης αυτής ήταν η ανάπτυξη μιας πολλαπλής τεχνικής μαγνητικών σφαιριδίων με 3 αντιγόνα [εκχύλισμα λιποπολυσακχαρίτη κυτταρικού τοιχώματος *Brucella* R φάσης (rLPS), ολόκληρο κύτταρο *Brucella* S φάσης (*B. suis* 1330 smooth) και ολόκληρο κύτταρο *Yersinia enterocolitica* O:9.] η οποία να διαχωρίζει τα *Brucella*

οροθετικά από τα *Brucella* οροαρνητικά ζώα και να ανιχνεύει τις διασταυρούμενες αντιδράσεις με την *Y. enterocolitica* O:9.

Χρησιμοποιήθηκαν 126 οροί οικόσιτων χοίρων οι οποίοι διαχωρίστηκαν σε 3 ομάδες. Η ομάδα Α (*Brucella* θετικοί) περιελάμβανε 29 ορούς από μολυσμένους και θετικούς στην καλλιέργεια χοίρους. Συγκεκριμένα, 25 από αυτά τα ζώα, προερχόμενα από την Ισπανία, ήταν μολυσμένα από το στέλεχος *B.suis* biovar 2 και τέσσερα, προερχόμενα από τη Νότιο Αμερική, ήταν μολυσμένα από το στέλεχος *B. suis* biovar 1. Η ομάδα Β (*Brucella* αρνητικοί) περιελάμβανε 80 τυχαία επιλεγμένους ορούς από χοίρους από τη Μεγάλη Βρετανία, η οποία είναι απαλλαγμένη από τη νόσο. Η ομάδα Γ περιελάμβανε 17 ορούς από κοπάδια της Μεγάλης Βρετανίας, που βρέθηκαν ψευδώς θετικοί, ύστερα από εξέταση με κοινές ορολογικές τεχνικές. Επίσης χρησιμοποιήθηκαν 49 οροί αγριόχοιρων από την Ισπανία, οι οποίοι διαχωρίστηκαν σε 2 ομάδες. Η ομάδα Α (*Brucella* οροθετικοί) περιελάμβανε 18 ορούς, ενώ η ομάδα Β (*Brucella* οροαρνητικοί) περιελάμβανε 31 ορούς.

Το αντιγόνο rLPS ανίχνευσε 26 από τους 29 *Brucella* θετικούς οικόσιτους χοίρους (23/25 μολυσμένους από *B. suis* biovar 2 και 3/4 μολυσμένους από *B.suis* biovar 1), ενώ όλοι οι ψευδώς θετικοί οροί (17/17) και 75/80 *Brucella* αρνητικοί οροί ήταν αρνητικοί. Το ίδιο αντιγόνο ανίχνευσε 15/18 οροθετικούς αγριόχοιρους, ενώ ήταν αρνητικό για όλους τους οροαρνητικούς (31/31). Το αντιγόνο *B. suis* 1330 ανίχνευσε όλους τους θετικούς οικόσιτους χοίρους (29/29), ήταν αρνητικό για όλους τους αρνητικούς (80/80), αλλά ήταν θετικό για 13/17 ψευδώς θετικούς ορούς. Στους αγριόχοιρους, το ίδιο αντιγόνο ανίχνευσε όλους τους οροθετικούς (18/18) και ήταν αρνητικό για 30/31 οροαρνητικούς. Τέλος, η αναλογία των τιμών διάμεσης έντασης φθορισμού (MFI) μεταξύ του αντιγόνου *B. suis* 1330 και του αντιγόνου της *Y.* enterocolitica O:9 διαχώρισε πλήρως τους *Brucella* θετικούς οικόσιτους χοίρους από τους ψευδώς θετικούς (ευαισθησία:100%, ειδικότητα:100%).

Τα αποτελέσματα αυτά δείχνουν τη δυνατότητα εφαρμογής της πολλαπλής τεχνικής μαγνητικών σφαιριδίων για την επιτήρηση της βρουκέλλωσης στους οικόσιτους χοίρους και στους αγριόχοιρους, καθώς και την ικανότητά της να ανιχνεύει τη διασταυρούμενη αντίδραση με την *Y. enterocolitica* στους οικόσιτους χοίρους.

Κεφάλαιο 4 – Η διαγνωστική αξία μίας πολλαπλής τεχνικής μαγνητικών σφαιριδίων (multiplex bead assay) και μίας τεχνολογίας μικροσυστοιχιών (microarray technology) ως οροδιαγνωστικών εργαλείων για την ταυτόχρονη ανίχνευση αντισωμάτων έναντι των παθογόνων Mycobacterium bovis, Brucella suis and Trichinella spiralis στους αγριόχοιρους.

Ο σκοπός της μελέτης αυτής ήταν να εκτιμηθεί η διαγνωστική αξία δύο πολυσύνθετων τεχνικών, μίας πολλαπλής τεχνικής μαγνητικών σφαιριδίων (multiplex bead assay) και μίας τεχνολογίας ορολογικών μικροσυστοιχιών (serology microarray), για την ταυτόχρονη ανίχνευση αντισωμάτων έναντι των παθογόνων *Mycobacterium bovis*, *Brucella suis* and *Trichinella spiralis* και να συγκριθούν τα αποτελέσματά τους με δύο συμβατικές τεχνικές ανοσοενζυμικής ανοσοπροσρόφησης ("in house" ELISA), μία για τη φυματίωση και μία για τη βρουκέλλωση, καθώς και με μία εμπορικά διαθέσιμη ELISA για την ανίχνευση αντισωμάτων κατά της τριχινέλωσης στους αγριόχοιρους.

Εκατόν εξήντα εννιά οροί αγριόχοιρων από την Ισπανία με γνωστό ορολογικό προφίλ φυματίωσης (64 θετικοί, 105 αρνητικοί) χρησιμοποιήθηκαν για την ανάπτυξη και την εκτίμηση της διαγνωστικής αξίας του multiplex bead assay και την εκτίμηση της διαγνωστικής αξίας του serology microarray. Για τον ίδιο σκοπό, χρησιμοποιήθηκαν 68 οροί αγριόχοιρων από την Ισπανία με γνωστό ορολογικό προφίλ για την βρουκέλλωση (29 θετικοί, 39 αρνητικοί) και 118 οροί αγριόχοιρων από την Ισπανία και την Ελλάδα με γνωστό ορολογικό προφίλ για την τριχινέλλωση (21 οροθετικοί, 97 οροαρνητικοί).

Τα αντιγόνα που χρησιμοποιήθηκαν για την ανάπτυξη και την εκτίμηση της διαγνωστικής αξίας του multiplex bead assay ήταν ένα ανασυνδυασμένο αντιγόνο MPB83 για τη φυματιώση, ολόκληρο κύτταρο *Brucella* S φάσης (*B. suis* 1330) για τη βρουκέλλωση και το E/S *T. spiralis* για τη τριχινέλλωση. Επιπλέον, εκτιμήθηκε η διαγνωστική αξία μίας ήδη ανεπτυγμένης τεχνολογίας ορολογικών μικροσυστοιχιών (serology microarray), η οποία περιείχε 116 διαφορετικά αντιγόνα, μεταξύ των οποίων ήταν και τα παραπάνω, για την ανίχνευση αντισωμάτων έναντι 63 ιϊκών, βακτηριακών και παρασιτικών παθογόνων. Λόγω του ότι δεν υπάρχουν εμπορικά

διαθέσιμες ELISA για την ανίχνευση αντισωμάτων έναντι της βρουκέλλωσης και της φυματίωσης των αγριόχοιρων, αναπτύξαμε στο εργαστήριο δυο τεχνικές ELISA ("in house" ELISA), χρησιμοποιώντας στη μία το αντιγόνο MPB83 και στην άλλη το *B. suis* 1330. Τα αποτελέσματα του κάθε αντιγόνου (MPB83, *B. suis* 1330 , E/S *T. spiralis*) συγκρίθηκαν μεταξύ των ορολογικών τεχνικών που χρησιμοποιήθηκαν (πολλαπλή τεχνικής μαγνητικών σφαιριδίων, τεχνολογίας ορολογικών μικροσυστοιχιών, ELISA), καθώς και μεταξύ των ορολογικών τεχνικών και των μεθόδων αναφοράς.

Η πολλαπλή τεχνική μαγνητικών σφαιριδίων διαχώρισε τους οροθετικούς στη φυματίωση αγριόχοιρους από τους οροαρνητικούς με ευαισθησία 98,4% και ειδικότητα 85,7%, ενώ οι αντίστοιχες τιμές για τη βρουκέλλωση ήταν 100% και 97,4% και για την τριχινέλλωση 90,5% και 99%. Ομοίως, η ευαισθησία και η ειδικότητα τεχνολογίας ορολογικών μικροσυστοιχιών ήταν ικανοποιητικές (92% και 92,4% για τη φυματίωση, 100% και 91,7% για τη βρουκέλλωση και 75% και 98,7% για την τριχινέλλωση, αντίστοιχα). Τα αποτελέσματα και των δύο τεχνικών δεν διέφεραν στατιστικά από τις μεθόδους αναφοράς, με μόνη εξαίρεση τα αποτελέσματα της πολλαπλής τεχνικής μαγνητικών σφαιριδίων για τη φυματίωση, αλλά ούτε από τα αποτελέσματα της ELISA.

Κεφάλαιο 5 – Καταληκτικά σχόλια και μελλοντικές έρευνες

Στο κεφάλαιο αυτό συνοψίζονται οι λόγοι για τους οποίους είναι απαραίτητη η διαρκής παρακολούθηση της έκθεσης των αγριόχοιρων σε διάφορους παθογόνους παράγοντες, σε εθνικό επίπεδο, καθώς και η διαγνωστική αξία της πολλαπλής τεχνικής μαγνητικών σφαιριδίων και της τεχνολογίας ορολογικών μικροσυστοιχιών.

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Chapter 1: Introduction

1.1 Wildlife-Wild boar

The wide distribution of wild animals throughout the world, in combination with the emergence of infectious pathogens originating from them, have a substantial impact on human health, livestock healthiness and productivity, agricultural production and wildlife conservation (Artois et al., 2001; Gortazar et al., 2006). In recent decades, factors such as human habitation to suburban areas, increased use of lands for agricultural purposes, increased hunting activities and consumption of wild animals' meat, environmental changes that alter the distribution of wild hosts and vectors, contribute to a higher contact rate between wildlife and humans or/and domestic animals. Worth noting that 60.3 % of the emerging or re-emerging diseases recorded in the past decade were caused by zoonotic disease agents and 71% of the pathogens involved were of wildlife origin (Cunningham, 2005; Jones et al., 2008). Two different patterns of transmission of pathogens from wild animals to humans have been witnessed (Bengis et al., 2004); the transmission of pathogens whose main hosts responsible for maintenance and transmission are the humans (e.g. influenza A, Ebola virus) and the direct or vector-mediated, transmission of animal pathogens (e.g. rabies, West Nile virus). Wildlife reservoir dynamics involve various host species and populations that may vary in their contribution to pathogen transmission and persistence over space and time. By "reservoir host" is designated one or more epidemiologically connected populations or animal species in which the pathogen can be permanently maintained and from which it is transmitted to a defined target population (Haydon et al, 2002). Among wildlife, wild boar is considered one of the major reservoir hosts not only in Europe, but worldwide.

Wild boar (*Sus scrofa*) is indigenous in many countries, worldwide. In recent decades, investigations have documented an increase of their population in Europe (Saez-Royuela et al., 1986; Laddomada, 2000). Wild boar is one of the most popular big-game species in Greece and their population number is estimated to be approximately 19.033 individuals that are widely distributed all over the continental part of the country (Tsachalidis and Hadjisterkotis 2009). Furthermore their population density has increased in many areas, especially those with shrub lands, agroforesrty formations

in combination with cereal cultivations (Birtsas and others 2007, Giannakopoulos 2012). The overabundance of these animals represents a relevant risk factor for transmission of pathogens to domestic animals and humans (Gortazar et al., 2007). Worldwide, wild boar seems to play a direct or indirect role in the circulation, maintenance and transmission of important emerging and re-emerging pathogens for both domestic pigs (e.g. porcine circovirus-2, porcine reproductive and respiratory system virus, Aujesky's disease virus, *Brucella suis*, *Mycobacterium bovis*, *Trichinella* spp) and humans (e.g. Hepatitis E virus, *B. suis*, *M. bovis, Trichinella* spp). In Greece although there are many surveys indicating the role of domestic pigs on the transmission of these pathogens, to the best of our knowledge, there is only sparse information on the role of European wild boar (Sofia and others 2008; Billinis 2009).

1.2 Infectious diseases of wild boar

1.2.1 Brucellosis

Brucellosis, is an important infectious disease in the swine industry caused by the Gram negative, facultative intracellular pathogen, *B. suis*. This species is divided into 5 biovars and only biovars 1, 2 and 3 are responsible for brucellosis in domestic pigs. Among these three biovars, biovar 2 is usually the causative agent of porcine brucellosis in Europe, whereas biovar 1 is mainly present in North and South America, Asia, and Oceania and biovar 3 in China, United States and only occasionally in Europe (Cvetnic et al., 2009; Garcia-Yoldi et al., 2007; OIE, 2013;). Although biovar 2 has been isolated from humans (Teyssou et al., 1989; Paton et al., 2001; Garin-Bastuji et al., 2006), it is considered weakly zoonotic in contrast with biovars 1 and 3 (Godfroid et al., 2005), which cause severe disease in humans (Munoz et al., 2010; OIE 2013). *B. suis* biovar 2 is widely spread among European wild boar (*Sus scrofa*) populations (Munoz et al., 2010), as it has been isolated from these animals in many countries of Central and Western Europe, enhancing the belief that they can represent a possible source of infection for domestic pigs and humans (Meng et al., 2009; Munoz et al., 2010). The

bacterium may spread to domestic pigs via interaction with infected wild boar and to humans via the handling and/or consumption of raw or undercooked wild boar meat.

The diagnosis of brucellosis is based on either the isolation-identification of the bacterium (direct diagnosis) or on the detection of the immunologic response of the swine host (indirect diagnosis). Specifically, the direct diagnosis includes the microscopic examination (Stamp's stained smears from vaginal swabs, placentas, aborted foetuses or lymph nodes), the bacterial culture (aborted foetuses or dead piglets, foetal membranes, vaginal secretions, milk, semen, arthritis or hygroma fluids, the late pregnant or early post-parturient uterus, udder, liver, kidney, testes, epididymides, vesicular glands, prostate and bulbourethral glands) and the molecular techniques such as PCR, real-time PCR, Restriction Fragment Length Polymorphism (RFLP), Variable Number of Tandem Repeats (VNTR), northern blots, sequencing of complementary DNA (cDNA) libraries, serial analysis of gene expression (SAGE) and microarrays including cDNA oligonucleotide arrays for Microbial Diagnostic Micro-arrays (MDMs) (OIE 2013; EFSA 2009). Although these methods may confirm the presence of the pathogen, the low sensitivity and specificity of microscopic examination, the technical difficulties, danger of laboratory personnel infection and low sensitivity of culture when samples from live animals are used, and the fact that molecular methods using readily available samples (blood, serum, etc) have not been fully validated, make them inappropriate for routine use.

The indirect diagnostic tests include brucellin skin test and serology techniques such as the Rose bengal test (RBT), the indirect and the competitive enzyme-linked immunosorbent assay (iELISA and cELISA, respectively), the serum agglutination test (SAT), the complement fixation test (CFT) and the fluorescent polarisation assay (FPA). Although none of the above serological tests is considered reliable for the diagnosis in individual swine (OIE 2013), factors such as the easily accessible samples (blood), their relatively low cost and high sensitivity, along with the efficacy of eradication programs of brucellosis that have been based on serological testing, motivate scientists to improve their diagnostic value and make some of them suitable for routine testing.

All the smooth *Brucella* spp, like *B. suis*, react in agglutination tests with antisera raised against smooth *Brucella* species. The outer membrane of *B. suis* is composed of

phospholipids, proteins and smooth lipopolysaccharide (sLPS). The sLPS is composed of the lipid A, the core oligosaccharide, and the O-polysaccharide (OPS or O-antigen or O-side chain domains). The most commonly used serological assays (RBT, CFT, iELISA) have been developed to detect antibodies against the sLPS antigen (OIE, 2013). The acceptable sensitivity of these assays is due to the immunodominance of the sLPS O-antigen. On the other hand, the closely related structures of *Brucella* spp and *Yersinia enterocolitica* O: 9 sLPS O-antigens (Meikle et al., 1989; Cloeckaert et al., 1998; Kittelberger et al., 1998; Jungersen et al., 2006; Nielsen et al., 2006) reduce the specificity of the above assays, resulting in *Brucella* false positive serological reactors (FPSR). Experimental studies showed that the replacement of the O-side chain dependant antigens by rough *Brucella* LPS antigens which consist only of the Lipid A and core sugars gave satisfactory and promising results for the resolution of the problem of FPSR (McGiven et al., 2012). Other antigens that have been used for the serodiagnosis of porcine brucellosis are the outer membrane proteins (OMP), which are also expressed on the surface of *B. suis* but lack sensitivity compared with the sLPS-based serological tests (EFSA 2009).

Unfortunately, there are no brucellosis tests validated for wild boar. In the most epidemiological studies the prevalence of *Brucella* spp in wild boar populations has been determined by the use of classical serological tests such as RBT or of commercially available ELISAs which have been validated for domestic pigs (Al Dahouk et al., 2005; Koppel et al., 2007). Recently a multi-species iELISA using *Brucella* sLPS antigen has been developed and validated to access brucellosis prevalence in wild ungulates in Spain (Munoz et al., 2010).

The vital role of the serological assays to the surveillance and monitoring of *B. suis* necessitates the development and validation of novel technologies, capable of screening simultaneously many serum samples against a variety of *Brucella* antigens, minimizing the number of intervention steps and reducing the "hand on" time without compromising their diagnostic value. The already existing techniques that permit the automation standards are the ELISA (96-well format), the FPA and the time-resolved fluorescent resonance energy transfer (TR-FRET) formats, which have been evaluated for brucellosis (McGiven, 2013). Also multiplexed techniques, such as antigen

microarrays and bead-based assays, could be useful tools for *Brucella* serology in domestic pig and wild boar.

1.2.2 Tuberculosis

Bovine tuberculosis is a chronic granulomatous bacterial disease caused by *M. bovis*, which can infect not only a wide range of domestic mammals, but also wildlife and humans (de Lisle et al., 2002; Grange, 2001) and has important impact on the economy and public health (Corner, 2006). Many countries, including Greece, have successfully eradicated bovine tuberculosis. The failure of the eradication programs and the re-emergence of the disease in countries such as United Kingdom, Ireland, and Spain have been associated with the occurrence of tuberculosis in wildlife reservoir species (Bengis, 1999; de Lisle et al., 2002). Consequently, disease surveillance and control measures are required for both livestock and wildlife. Wild boar is one of the species which are implicated in the maintenance and the transmission of *M. bovis* in Europe (Naranjo et al., 2008; Gortazar et al., 2011).

The methods that can be used for the diagnosis of bovine tuberculosis include the isolation-identification of *M. bovis* (microscopic examination, culture, molecular methods), the tuberculin skin test and the blood-based laboratory tests. Post-mortem diagnosis such as lesion detection in slaughter, culture and strain typing, usually lacks sensitivity, although genotyping of bacterial isolates or PCR products is increasingly becoming essential for disease control and eradication (Schiller et al., 2010). On the contrary, ante-mortem diagnostic tests such as the tuberculin skin test, the gamma-interferon assay and serological detection of specific antibodies are used more frequently with promising diagnostic performance. Specifically, the eradication programs of tuberculosis in cattle are based on the intra-dermal tuberculin test and slaughter policy. The intra-dermal tuberculin test is recognized as the standard screening test for the detection of bovine tuberculosis (OIE, 2009) and involves the intra-dermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of a local delayed hypersensitivity reaction. Gamma interferon

assay, which also measures cellular immunity, is also approved in the European Union and it is used as an ancillary test in order to maximize the detection of infected animals or to confirm the result of intra-dermal test (OIE, 2009; Bezos et al., 2014). The tuberculin test and the gamma interferon assay are not easily applicable for the detection of tuberculosis in wild boar population, because the first requires handling of the animal and the latter the use of fresh blood samples. Furthermore, these tests have not been fully validated for species other than cattle (Monaghan et al., 1994). On the contrary, the use of serological assays seem to be an efficient alternative tool for screening wild boar for *M. bovis* infections (Aurtenetxe et al., 2008; Lyashchenko et al., 2008; Boadella et al., 2011).

Previous studies have shown that *M. bovis* infection of cattle elicits an early and robust cell-mediated response and a peculiarly weak antibody response (Pollock et al., 2001; Welsh et al., 2005), but, as the infection progresses, immunodominant antigens elicit a detectable humoral response. The bacterial antigens which are released in larger amounts are the MPB70 and MPB83. Experimental studies have shown that antibodies to MPB70 develop after 18-22 months of infection, whereas anti-MPB83 antibodies may be detected much earlier, around 4 weeks post-infection (Harboe et al., 1990; Fifis et al., 1994; Waters et al., 2006). Also, antibodies against the ESAT-6 antigen were detected 12 weeks after experimental infection (Lyashchenko et al., 1998). Although several antigens, such as ESAT-6, CFP-10, PPD, MPB70, have been used to detect *M. bovis*-specific antibodies, the highest and earliest humoral response is detected with the MPB83, not only in domestic livestock, but also in wild animals (Amadori et al., 1998; Lilenbaum et al., 1999; McNair et al., 2001; Amadori et al., 2002; Lyashchenko et al., 2008; Wiker, 2009). Finally, several studies have examined the value of chimeric proteins (formed by the combination of multiple antigens) for the serodiagnosis of tuberculosis, showing a sensitivity over 65% and a specificity of 95% (Liu et al., 2007; Waters et al., 2011; Souza et al., 2012). Considering the application of serological tests for tuberculosis in wild boar, the fact that different animal species respond to different bacterial antigens and that test accuracy may be impacted by environmental and host factors such as disease prevalence, nutrition, handling, stress, environmental mycobacterial exposure and bacterial burden, highlights the importance of using

samples from appropriate and carefully selected animal populations (Schiller et al., 2010).

1.2.3 Trichinellosis

Trichinellosis is caused by parasitic nematodes of the genus *Trichinella* and is one of the most important zoonotic diseases, worldwide (Gibbs, 1997; Murrell et al., 2000; Pozio, 2007). The serious impact of the disease on porcine production and food safety is the basic reason that many countries have eradicated it or are trying to control it. Human infection occurs after the ingestion of *Trichinella* larvae that are encysted in muscle tissue of domestic pigs or wild boar. The implementation of the latter along with red foxes in the sylvatic cycle of the parasite makes eradication of the disease difficult and complicated (Rafter et al., 2005; Pozio et al., 2009). Due to the *Trichinella* spp infection of wild boar and the common outbreaks of human trichinellosis associated with the consumption of their meat (Serrano et al, 1989; Rondriquez-Osorio et al., 1999; García-Sánchez et al., 2009) some countries have implemented monitoring programs, not only for domestic species but also for wild boar (EFSA, 2011).

The diagnosis of trichinellosis can be accomplished by direct and indirect methods. The former include artificial digestion, tissue compression (trichinoscopy) and molecular methods (PCR). Artificial digestion of muscle tissues is recommended for international trade and careful selection of muscle samples can increase its sensitivity because the preferred muscles vary among hosts. For example, in pigs, the preferred site for *Trichinella* larvae is the diaphragm, whereas in horses it is the tongue. The remaining direct methods (i.e. trichiniscopy and PCR), lack sensitivity (OIE, 2012). The indirect methods include the immunofluorescence assay (IFA), immuno-electrotransfer blot (IEBT), western blot, enzyme immunohistochemical assays, and ELISA, which detect anti-*Trichinella* antibodies not only in serum, but also in meat juice samples. Although they are not recommended for individual animal testing or for determining actual zoonotic risk, they can be used for the estimation of the level of exposure to *Trichinella* spp (Noeckler and Kapel, 2007). Additionally, serology is

considered to be suitable for the surveillance and epidemiological investigations of wildlife (Gamble et al., 2004).

Previous studies in pigs have shown that ELISA can detect infection levels as low as one larva per 100gr of tissue (Gamble et al., 2004). On the other hand, the lag time of the antibody response following the ingestion of larvae may decrease sensitivity and can explain the relatively low rate of false-negative results, since antibodies are detectable 3 – 5 weeks or more after exposure (Gamble, 1996). The diagnostic performance of the serological assays is mainly dependent upon the type and the quality of the antigen used. The predominant *Trichinella* antigen (TSL-1) is localized on the surface of the parasite's cuticle, in its stichocyte cells and it is secreted by the first-stage larvae in the muscle. Stichosome antigens collected from the excretory/secretory (ES) products of *Trichinella* muscle larvae and the synthetic carbohydrate antigens (tyvelose) are the most specific antigens for screening purposes. Nevertheless, the sensitivity of the ELISA using tyvelose is lower than using ES antigens (Gamble et al., 1997; Forbes et al., 2004). Additionally, the ES antigens are conserved in all species and genotypes (Ortega-Pierres et al., 1996) and can be used for detection of *Trichinella* both in pigs and wild boar (Kapel, 2001). Studies have shown that the serological assays are more sensitive than artificial digestion in pigs (Gamble et al., 2004) and wild boar (Gomez Morales et al, 2014), but may lack specificity due to false positive reactions (Frey et al., 2009; Nöckler et al., 2009). In any case, *Trichinella* serology is considered to be suitable for the surveillance and epidemiological investigations of wildlife, especially in countries which are considered trichinellosis-free.

1.2.4 Other significant bacterial and parasitic pathogens of wild boar

Actinobacillus pleuropneumoniae and *Mycoplasma hyopneumoniae* are the causative agents of significant respiratory diseases of swine, pleuropneumonia and enzootic pneumonia respectively, which are associated with economic losses due to reduced growth, increased mortality, secondary infections and treatment cost. Infection occurs generally

through direct contact with respiratory secretions from carrier animals, though airborne transmission has been also reported for both pathogens (Kristensen et al., 2004; Stark et al., 1998). In the last decades, there is an increase tendency for outdoor pig production permitting contact with wild animals such as wild boar. Therefore, there is a possibility of transmission of these pathogens between domestic or/and outdoor pigs and wild boar. Nevertheless, few surveys have been conducted in order to investigate the role of the latter as a reservoir for these pathogens (Vengust et al., 2006; Sibila et al., 2010; Baker et al., 2011; Kuhnert and Overesch, 2014). Identical genotypes of *M. hyopneumoniae* were found in wild boar and domestic pigs in Switzerland, indicating the possible transmission of the pathogen between these species (Kuhnert and Overesch, 2014). Regardless of the source of these pathogens (pigs or wild boar), the transmission may affect wildlife conservation and domestic pig productivity. The diagnosis is based on culture, molecular and serological methods. PCR seems to be the most accurate for the diagnosis of *M. hyopneumoniae* (Artiushin et al., 1993; Calsamiglia et al., 1999), but it is not practical for screening purposes in wild boar. There are commercially available ELISAs for the detection of antibodies against this pathogen but they have low sensitivity, perhaps because the bacterium remains attached on the ciliated epithelium of the respiratory track and there is low exposure to the immune system (Thacker, 2004). Nevertheless, the use of serological assays for the diagnosis of *M. hyopneumoniae* in wild boar is essential in order to control the disease. Regarding *A. pleuropneumoniae*, serological assays are the most valuable tools in assisting diagnosis in live animals (Rycroft and Garside, 2000). In the past, the presence of different serotypes of the bacterium complicated the serological diagnosis, as there were no tests capable of identifying and differentiating all serotypes. Lately, a commercially available ELISA for pigs with the use of the exotoxin ApxIV as antigen, which is produced by all 15 known serotypes, solved this problem.

Salmonella spp is the causative agent of the second most common zoonotic disease in Europe with important economic impact on pig industry (EFSA, 2010). The bacterium can infect a variety of domestic and wild animals, including wild boar (Vieira-Pinto et al., 2011). Recently, *S. typhimurium* and *S. enteritidis* were isolated from hunted wild boar in Italy, indicating their possible role as a source of food borne human infection (Chiari et al., 2013). Furthermore, wild boar is omnivorous species and

thus can be used as sentinels for the presence of the environmental *Salmonella* serotypes. The diagnosis of salmonellosis is based on the isolation of the bacterium (Christensen et al., 2002; EFSA 2010) and the detection of specific antibodies with assays, like ELISA. The latter is not recommended for individual diagnosis in pigs, but is appropriate for screening purposes (Wong et al., 2003; Nollet et al., 2005; EFSA 2010) and previous studies in pigs showed the efficiency of the LPS antigen for routine testing (Nielsen et al., 1995; Proux et al., 2000).

Toxoplasma gondii is an obligate intracellular parasite that infests all warm-blooded animals, including humans (OIE, 2008). The definite hosts are the cats and wild felids, while a variety of domestic and wild animals are intermediate hosts (Davidson 2000). Wild boar is exposed via consumption of food contaminated by sporulated oocysts. Human infection occurs after the ingestion of raw or lightly-cooked meat containing live cysts or of raw vegetables and after exposure to oocysts from cat faeces. Toxoplasmosis outbreaks have been reported in hunters, after consumption undercooked wild boar meat (Choi et al., 1997) and antibodies against *T.gondii* have been detected in hunters in Spain (Gauss et al., 2005; Ruiz-Fons et al., 2006). Therefore, wild boar could be an indicator of the transmission risk to human and domestic animals and should be included in the surveillance programs. The diagnosis of toxoplasmosis is based on the identification of the parasite in tissue sections, by immunochemistry and molecular methods or on the detection of antibodies in serum. The gold standard serological assay is the dye test, but it carries the risk for laboratory personnel infection (OIE, 2008). An alternative test, useful for screening purposes is indirect immunofluorescent antibody test (IFAT), which titers are usually comparable with that of the dye test.

Neospora caninum is an obligate intracellular parasite which affects a wide range of warm-blooded animals (Gondim, 2006; Dubey and Schares, 2011). Neosporosis is associated with severe economic losses in cattle due to abortions. Domestic dogs and some wild canids are the recognized definite hosts and they excrete oocysts to the environment. Although several studies have been carried out in order to demonstrate the role of wildlife in the circulation and the maintenance of the parasite in different regions, limited information exists on *Neospora caninum* transmission dynamics in wild

boar. Since the methods used for the identification of the parasite are impractical for screening purposes, many serological assays have been suggested, especially in cattle, such as IFAT, *Neospora* agglutination test (NAT), ELISA, cELISA, immunoblotting and western blot (von Blumröder et al., 2004; Wapenaar et al., 2007). Among them, IFAT is a well established test for detecting anti-*N. caninum* antibodies in various animal species (Almería et al., 2007).

1.2.5 Significant viral pathogens of wild boar

Aujeszky's disease, also known as pseudorabies, is caused by the *Suid herpesvirus* 1 and is one of the most important diseases worldwide. Domestic pig is the natural host of the virus, which can infect other mammals, including wild boar. The high seroprevalence rates in wild boar from several countries indicate the circulation and maintenance of the virus among this species (Gortazar et al., 2002; Lari et al., 2006; Gortazar 2007). The possibility of infection spread from wild boar to domestic pigs and vice versa is controversial, since some studies have shown that it is possible (Muller et al, 2001) and others not (Muller et al., 1997). The reference method for Aujesky's disease serology was initially virus neutralization test, which was later replaced by ELISA (OIE, 2012). A variety of commercially available ELISA kits for pigs could be used for the detection of antibodies against the virus in wild boar.

The porcine reproductive and respiratory syndrome (PRRS) virus is an Arterivirus that causes reproductive failure of sows and respiratory problems of piglets and growing pigs. Two major antigenic types of the virus exist, type 1 (European) and type 2 (American). The role of wild boar in the transmission of the virus to domestic pigs is unclear, as their seropositivity rates range from low to nil (Albina et al., 2000; Vicente et al., 2002; Vengust et al., 2006; Montagnaro et al., 2010; Boadella et al., 2012, Roic et al., 2012). Nevertheless, monitoring of the virus in these species is crucial, due to the increasing outdoor pig breeding. Methods such as viral culture, immunochemistry, and molecular tests are available, but the ELISA technique is the most suitable for screening purposes. There are many commercially available ELISA

kits, which have been validated in domestic pigs and can differentiate serological reactors to the European and the American types of the virus (OIE, 2010).

Porcine circovirus-2 (PCV-2) is one of the two PCV genotypes, which is associated with a disease called postweaning multisystemic wasting syndrome (PMWS) (Harding, 1996) with high economic impact on the pig industry (Segales et al., 2005a). Previous studies have shown that the virus circulates at a high rate among wild boar in Europe, affecting their mortality rates. Worth noting that identical PCV-2 isolates have been found in wild boar and domestic pigs of same or distinct regions (Knell et al., 2005; Csa´gola et al., 2006), but without determining the direction of transmission. The accurate diagnosis of PMWS in live animals is based on quantitative PCR using blood samples and/or tissue swabs (Brunborg et al., 2004; Olvera et al., 2004; Segalés et al., 2005b), which requires handling of the animals. As this is not easily applicable to wild boars, alternative methods can be used for diagnostic purposes. Although the ubiquitous nature of the virus advocates against the use of serology, many ELISA kits are commercially available for the detection of antibodies against PCV-2 in domestic pig sera.

Swine influenza is a highly contagious disease of pigs and is caused by type A influenza viruses (Olsen et al., 2006). The type A swine influenza viruses are further subdivided based on their haemagglutinin and neuraminidase proteins and the most frequently identified subtypes are the H1N1, H1N2, reassortant (r) H3N2, and rH1N2 (Choi et al., 2004; Marozin et al., 2002; Schrader & Süss, 2004; OIE, 2010). Previous studies showed that antibodies against the subtypes H1N1, H3N2 and H1N2 have been also detected in wild boar (Markowska-Daniel, 2003). The swine influenza virus is density-dependant and it could be endemic only in semi-captive or farmed animals. Nevertheless, monitoring of the disease in regions where wild boars interact with outdoor pigs or semi-captive wild boar is crucial. The primary serological test for the detection of antibodies against swine influenza virus in domestic pigs is haemagglutination inhibition, which requires paired sera collected 10-21 days apart. ELISA is the alternative and commonly used rapid test which has been described in literature and is also commercially available for pigs.

1.3 Novel technologies as serodiagnostic tools for wild boar pathogens

Wild boar may be used as sentinels for the surveillance and the monitoring of various emerging or re-emerging diseases important for livestock and human health. The development of novel technologies, such as microarrays and multiplex bead assay, has the potential to improve the efficiency of monitoring and surveillance of diseases in wild boar, allowing simultaneous screening of many samples for more than one pathogen. These assays could be used as serological tools, replacing the available ELISA-based tests, which are time-consuming and require large quantities of both sample and reagents, thus limiting their application for mass pathogen screening (Kricka, 1993; Silzel et al., 1998).

1.3.1 Microarray technology

Microarray technology is a tool for large-scale and high-throughput biological testing, as it is capable of detecting many addressable elements in a single experiment. A microarray consists of hundreds or thousands of spots containing specific oligonucleotides, cDNAs, proteins, peptides or other small molecules which have been chemically attached to a substrate and organized in a grid on a glass slide, plastic slide, silicon chip, fibre-optic array or filter membrane. Multiple studies have shown the great potential of microarrays in basic research, gene discovery, diagnostics, drug discovery and toxicological research.

Antigen microarrays are potential tools for the detection of humoral immune response in the setting of health and disease in humans, as these assays have been used to profile antibody reactivity during infections, to identify autoantibodies in autoimmune diseases (Quintana et al., 2012), IgE antibodies in allergies (Sanz et al., 2011), humoral responses against neoplastic antigens (Wang et al., 2005) and post-vaccination antibody production (Neuman de Vegvar et al., 2003). Many microarrays have been developed for the detection of antibodies, using various types of disease-

specific antigens, not only in humans (Wadia et al., 2011; Papp and Prechl, 2012; Liang et al, 2013), but also in domestic animals, like chickens and pigs (Freidl et al., 2014; Meyer et al., 2015). In general, the antigen microarray procedure requires reproducible "printing" of sub-millimeter antigen spots on a small surface, usually a microscopic slide, treatment of the array of the antigens with the tested serum and detection of bound antibodies using the available image analysis software. They can measure hundreds of antigen-antibody interactions in parallel using a small amount of sample. The challenge that this assay faces is that it should detect antibodies with different specificities against a variety of antigens, which may exist at different or similar concentrations. Nevertheless, antigen microarrays seem to have the same efficiency as ELISAs to detect antibodies against infectious agents both in humans and animals (Mezzasome et al., 2002; Becarese-Hamilton et al., 2004; Meyer et al., 2015). Furthermore, microarrays can be designed to include several replicates of each antigen which may be used to improve reproducibility and diagnostic accuracy as the antibodies can be detected even in the case of failure of one of the antigens (Petric 2010). This miniaturized parallelized immunoassay can be used as a surveillance tool in wildlife, collecting a maximum of diagnostically relevant information with small quantities of samples and reagents. To the best of our knowledge, microarrays have been developed for the detection of the presence or absence of genetic sequences characteristic of specific pathogens in wild boar and for gene expression profiling of wild boar infected with various bacterial pathogens (Galindo et al., 2009; Meemken et al., 2013; Kraushaar and Fetch, 2014), but antigen microarrays have not been used to detect humoral immune responses against infectious diseases in these animals.

1.3.2 Multiplex bead assay

Multiplex bead assays can be used for the simultaneous detection and quantification of multiple analytes in a solution, employing microbeads as a solid support. A variety of ligands, such as antigens, antibodies, oligonucleotides, enzyme substrates or receptors, can be captured on these microbeads. The technique relies on the use of different bead sets, which are distinguished from each other by the varied amounts of 1–3 fluorescent

dyes, their size and/or their shape. Each of bead set is coated with a specific ligand, allowing the use of multiple ligands in one reaction and thus the simultaneous detection of up to 500 different analytes per sample.

Surveys have been carried out using multiplex bead assay as a serodiagnostic tool in humans (Antonsson et al., 2010; Casabonne et al., 2009; Dias et al., 2005) and animals (van der Wal et al., 2012; Anderson et al., 2011; Watson et al., 2009; Perkins et al., 2006), in which the ligands that have been used include host immune proteins, such as antibodies and cytokines, and viral, bacterial or parasitic antigens. The beads which can be used are fluorescent polysterene beads or magnetic beads. After antigen-antibody interaction, a biotinylated secondary antibody is used in order to identify the immune complexes and to react with a fluorescent marker, such as phycoerythrin-streptavidin complex. The median fluorescent intensity of this antigen-antibody interaction is determined by the use of specific instruments. Two lasers excite each bead; the one identifies each bead's spectral address and the other quantitates the captured analyte. The main advantage of the multiplex bead assay, compared to ELISA, is the small volume of samples that is needed to measure multiple analytes simultaneously. Additionally, the multiplex bead assay can be performed within a 96-well microtiter plate, like ELISA, allowing the evaluation of a large number of samples and large number of analytes, reducing cost and labor. The fact that multiplex assay can be customized for the user's specific needs based upon the specific analytes of interest each time makes it a potential tool for screening purposes. Regarding its diagnostic performance, studies in animals have shown that it is comparable to (Christopher-Hennings et al., 2013) or even more sensitive than ELISA (Giménez-Lirola et al., 2012). Although many studies have been based on the development of multiplex bead assays for pig diagnostics (Lin et al., 2011; Bokken et al., 2012; Langenhorst et al., 2012; Giménez-Lirola et al., 2012;Crombé et al., 2013; van der Wal et al., 2013;), none has been developed for wild boar, to the best of our knowledge.

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Chapter 2: A serosurvey for selected pathogens in Greek European wild boar

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2.1 Introduction

In the last decades, an increase of wild boar population in Europe has been documented (Sáez-Royuela and Tellería 1986, Laddomada 2000). In Greece, wild boar is a native species with a wide distribution in all over the continental part of the country (Tsachalidis and Hadjisterkotis 2009). Recently, their population density has increased in many areas, especially those with shrub lands, agroforestry formations in combination with cultivations (cereals) (Birtsas and others 2007, Giannakopoulos 2012). Wild boar is one of the most popular big-game species in Greece with an increasing interest from hunters and can be found in many habitat types.

As the number of European wild boar increases, the interaction with domestic livestock also increases, and this raises concerns of direct and indirect human exposure to zoonotic agents (Gortázar and others 2007). Wild boar may represent reservoir of a long list of viral, bacterial and parasitic agents and may play a direct or indirect role on the circulation, maintenance and transmission of infectious diseases to domestic pigs. For example, wild boar are considered a limiting factor for the eradication of infectious diseases with significant economic impact in swine industry such as Aujeszky's disease virus (ADV) or porcine circovirus type 2 (PCV-2) (Meng and others 2009). Furthermore, the interface of humans with boars via hunting or agricultural purposes or consumption of infected wild boar meat is the basic reason that creates the appropriate circumstances for the transmission of importance for the public health pathogens such as trichinellosis or toxoplasmosis (Meng and others 2009). Control of wild boar diseases requires sound epidemiological information in order to investigate the prevalence of their exposure to various pathogens and to determine their geographical distribution in a country before the application of control measures (Morner and others 2002). Geographical information systems (GIS) represent a modern tool for surveillance of wildlife diseases and offer spatial analysis, which is an essential component of modern disease surveillance systems (Pfeiffer and Hugh-Jones 2002). GIS can be used to correlate environmental, climatic, socio-economic and many other data and factors with the prevalence of exposure to the infectious agent(s) in question. Although there are many surveys indicating the role of domestic pigs on the

transmission of pathogens in Greece, to the best of the authors' knowledge, there is only sparse information on the role of European wild boar (Sofia and others 2008, Billinis 2009).

The aim of this study was to investigate the seroprevalence for 10 selected pathogens, important for livestock and/or public health, in wild boar from different areas of Greece and to correlate results with environmental factors within a GIS.

2.2 Materials and methods

In collaboration with the Hunting Federation of Macedonia and Thrace, blood samples were collected from 94 free-ranging European wild boar from different areas of Greece during the hunting seasons 2006–2010. Hunters collected the blood samples by heart puncture or from the body cavities, and they stored them into sterile tubes at 4°C until arrival to the laboratory, within 24 hours. Sera were separated after centrifugation and were stored at −20°C pending examination.

Sera were tested for antibodies against PCV-2 using a commercial indirect ELISA (Ingezim Circo IgG, Ingenasa, Madrid, Spain). Also, ELISAs were used for the detection of antibodies against porcine reproductive and respiratory system (PRRS) (Porcine Reproductive and Respiratory Syndrome Virus Antibody Test Kit, IDEXX Laboratories, Westbrook, USA), ADV (PRV/ADV Gb Ab Test, IDEXX laboratories, Westbrook, USA), IA viruses (Ingezim Influenza Porcina, Ingenasa, Madrid, Spain), *Actinobacillus pleuropneumoniae* (APP-Apx IV Ab Test, IDEXX laboratories, Westbrook, USA) and *Mycoplasma hyopneumoniae* (M. hyo Ab Test, IDEXX, Westbrook, USA). Detection of antibodies against *Salmonella* species was done by an ELISA (Swine *Salmonella* Ab Test, IDEXX, Westbrook, USA), which detects antibodies against a broad range of *Salmonella* serogroups. Antibodies against *Trichinella* species were determined using a commercial kit (ELISA *Trichinella* Serum Screening, Institut Pourquier, Montpellier, France) that has been validated for wild boar, and it is based on the

excretory/secretory antigen of the parasite. All the above ELISAs were performed following the manufacturers' recommendations.

Finally, anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies were detected by indirect fluorescence antibody test kits using commercially available slides coated with parasite tachyzoites (Fuller Laboratories, Fullerton, California, USA) and antiporcine IgG conjugate (Porcine IgG FITC conjugate, VMRD Inc) was used. Serum samples were tested at twofold dilutions in PBS, starting from 1:40 (cut-off titre) until reaching the end-point titre.

The area from where all the 94 samples were obtained was located in the field using handheld Global Positioning System units or using longitude and latitude information provided by the hunters on Google Earth software (https://earth.google.com/). GIS layers were created to represent the geographic locations of the wild boar serum samples and of the free-ranging swine farms. The environmental variables for this study were derived from two main database categories: altitude and land cover. Altitude was extracted from a digital elevation model with a spatial resolution of 1 km2 (http://srtm.csi.cgiar.org/Index.asp) and land use were derived from the Corine Land Cover 2006 database (European Environment Agency, www.eea. europa.eu/data-and-maps). These data sets were converted to a common projection (Greek Grid projection system), map extent and resolution prior to use. ArcGIS V.10.1 GIS software (ESRI, Redlands, California, USA) was employed for description and analysis of spatial information. Cluster analysis for the seropositivity to at least one of the examined pathogens was performed with the Hot Spot Analysis tool that calculates the Getis-Ord Gi* statistic (Mitchell 2005).

Data on wild boar population density in each regional unit were gathered through a questionnaire survey of local Game offices of Forest services, Federal Rangers and members of local hunting clubs. An index with six classes of population density was created using the data of this survey. Moreover, the authors also carried out 112 interviews (76 federal rangers, 6 scientific collaborators of the Hunting Federation of Macedonia and Thrace, 20 heads of wild boar hunters and members of

local hunting clubs and 10 local Game offices of Forest services). Interviews were targeted to determine current wild boar presence and the estimated local population size. Reported data were plotted on Google Earth software.

The relationship between wild boar sex and seropositivity to each pathogen was examined with the Phi coefficient (Cheetham and Hazel 1969).

The authors examined the relationship between seropositivity to each particular pathogen and selected environmental variables (altitude, distance from the nearest free-ranging swine farms, land use, land cover) and the density of wild boar population. Because the first two variables were continuous, the hypothesis was tested with independent samples t test or, whenever the counts of seropositive or seronegative animals were less than five, with the non-parametric equivalent Mann-Whitney U test (Bradley 2007). The latter test was also used to check for possible relationship between seropositivity and wild boar population density; in addition, the authors used the Kendal tau correlation measure, which is suitable for comparing two categorical variables. Considering the environmental variables land use and land cover, the authors used the uncertainty coefficient, which is a measure for testing the relationships between two nominal variables, when one of them is considered a dependent variable (Fowler and others 2013). The analysis was performed with IBM SPSS V.22.0 (Gray and Kinnear 2012), and the results were considered significant when $P \leq 0.05$. The authors also used the Cramer's V measure in order to compare the seroprevalences between the mountain ranges A, B and C.

2.3 Results

The number of positive samples for each pathogen and distance between seropositive animals and closest freeranging swine farm are shown in Table 1. The locations where viral pathogen-seropositive wild boar samples have been collected are presented in Fig 1, while the same information for bacterial and parasitic pathogens is shown in Fig 2. Among the various pathogens examined, seropositivity was more common against *A.*

pleuropneumoniae, followed by ADV, PCV-2 and PRRS virus, while antibodies against the remaining agents examined were detected in < 10 per cent of the samples. None of the sera tested was positive for antibodies against *M. hyopneumoniae* (analytical results per sample in online supplementary file 1).

Figure 3 shows the origin of seropositive and seronegative wild boar samples included in the present study. The Gi* statistic for each feature in the data set appears as a z-score. The latter revealed a cluster (hot spot) of seropositivity to at least one of the pathogens examined near the Bulgarian borders (see online supplementary fig S1).

The mean altitude where seropositive wild boar samples have been obtained was 839.5 m above sea level (range 175–1720 m; sd 334.94 m). Most of them have been collected in tree cover, broadleaved, deciduous, closed forests, followed by shrub cover, closed-open, evergreen forests and fewer seropositive samples have been collected in agroforestry formations and cultivated land. Moreover, 855 free-ranging swine farms were present in the areas of the country inhabited by wild boar.

The seropositive wild boar have been hunted in regional units with medium or high wild boar population density according to the GIS population index layer (Fig 4). The highest mean density of hunted animals was observed in the Ioannina regional unit (1.5 ind/km^2) and the lowest in the Grevena regional unit (0.17 ind/km^2).

Although sex was known for only 51 out of the 94 wild boars, there was a slightly significant relationship with seropositivity to ADV (p=0.046), which was higher in females; 52.6 per cent of the females were seropositive versus 25 per cent among males.

There were no significant relationships among environmental factors (altitude, distance from the nearest free-ranging swine farms, land use, land cover) or density of wild boar population and seropositivity with the only exception of PRRS virus where a borderline association with land cover (P=0.05) was found (higher seropositivity in cultivated and managed areas). The above relationships were not apparent when the authors used the Bonferroni correction due to multiple testing, but they note them as indications of a possible interdependence. Also, Cramer's V measure was significant, taking into account the Bonferroni correction, in two cases: PRRS (Cramer's V=0.387,

P=0.001) and ADV (Cramer's V=0.287, P=0.021). In both cases, seropositivity rate was higher in the mountain range B. Considering PRRS, seropositivity rates in the mountain ranges A, B and C were 7 per cent, 32.1 per cent and 0 per cent, respectively, whereas the corresponding figures for ADV were 37.2 per cent, 50 per cent and 13 per cent, respectively.

2.4 Discussion

This is the first expanding serological survey of Greek wild boar for antibodies against pathogens with economic and/or zoonotic importance. The results of this study highlight the possible role in the circulation of viral, bacterial and parasitic pathogens in Greece. The high seropositivity rates for PCV-2 (19.1 per cent), ADV (35.1 per cent) and *A. pleuropneumoniae* (57.4 per cent) are compatible with data from other European countries (Gortázar and others 2002, Vicente and others 2002, 2004, Ruiz-Fons and others 2006, Vengust and others 2006, Sedlak and others 2008, Montagnaro and others 2010, Boadella and others 2012b, Roic and others 2012). On the contrary, the detection of antibodies against PRRS virus in 12.8 per cent of the samples was rather unexpected as many surveys have shown that the seroprevalence rates in free-ranging wild boar are usually low to nil in contrast to farmed or semi-captive wild boar (Albina and others 2000, Vicente and others 2002, Vengust and others 2006, Montagnaro and others 2010, Boadella and others 2012b, Roic and others 2012). Although seropositivity to PRRS virus may indicate contact between wild boars and domestic pigs, the intensive piggeries in Greece is not reachable to wild animals. Nevertheless, the latter is possible between wild boar and semi-captive pigs, taking into account that PRRS virus can be transmitted up to 4.7 km away from the source of contamination (Dee and others 2009).

There are indications that wild boar can play a role in the epidemiology of IA viruses, as antibodies against the three subtypes of swine influenza virus (SIV), H1N1, H3N2 and H1N2, have been detected in free and semicaptive wild boar (Ruiz-Fons and others 2008). In this study, seroprevalence was 1.1 per cent, whereas on a

global level varies from 0 per cent to 75 per cent (Saliki and others 1998, Gipson and others 1999, Vicente and others 2002, Vengust and others 2006, Kaden and others 2008, Sattler and others 2012). Every time that a high seroprevalence has been recorded, it applied to semicaptive or farmed animals, living at high density, which is a prerequisite for SIV to become endemic.

Antibodies against *Salmonella* species were detected in 4.3 per cent of the samples, which is compatible with the results from Spain (Vicente and others 2002). On the other hand, in some countries such as Italy (Montagnaro and others 2010) and Slovenia (Vengust and others 2006), seropositivity rates have been much higher (19.3 per cent and 47 per cent, respectively).

In countries where the density of wild boar is low and pig industry follows modern hygiene standards, *Trichinella* species transmission cycle is blocked (Pozio 2007). Although no human cases had been reported for decades in Greece (Sotiraki and others 2001), a medium (6.4 per cent) seropositivity rate was found in the present study compared with other European countries where seropositivity ranges from 0.11 per cent to 14 per cent (Pozio 2007, Hurníková and Dubinský 2009, Richomme and others 2010). Since health management is not applicable to wild boars, the seropositivity in this population may be of particular importance for the surveillance for this zoonotic agent, although the serological tests in free-ranging wild boar may overestimate its prevalence (Boadella and others 2012a).

Anti- *T. gondii* antibodies were found in 5.2 per cent of the samples, whereas the relevant figures in previous studies conducted in different European countries were 26.2 per cent for Czech Republic (Bártová and others 2006), 36.3 per cent for Spain (Ruiz-Fons and others 2006), 8.1 per cent for Slovak Republic (Antolová and others 2007), 23 per cent for France (Beral and others 2012) and 20.6 per cent for Portugal (Coelho and others 2014). Although the serological techniques used in the above studies, except one (Bártová and others 2006), differ from this study and thus a direct comparison of prevalences is not possible, these figures may be helpful to highlight the differences in the serological status of wild boar population against *T. gondii* across European countries. Gauss and others (2005) support that when wild boars live in

restricted areas and at high density they have an increased possibility of contact with oocysts.

The GIS analysis of the present study demonstrated that 855 from a total of >2000 free-ranging swine farms currently present in Greece are located inside the wild boar's distribution range, and this may represent an underestimate since there are some smaller farms inside the study area that may have not recorded. Home range size for wild boar varies from 4 to 31 km^2 , but they can move up to 6 km outside this area (Mailard and Fournier 1995, Sodeikat and Pohlmeyer 2007, Gaston and others 2008). Therefore, this study's data indicate that there is a substantial risk of mutual transmission of pathogens between the wild boar and the free-ranging swine populations.

Statistical analysis of the data showed that female wild boar were more frequently seropositive for ADV than the males. This female predisposition is similar with that reported in previous studies and has been attributed to the increased intraspecific contacts of females due to the younger age they reach sexual maturity and their social behaviour (Vicente and others 2005, Cano-Manuel and others 2014). The borderline association between the high PRRS seropositivity rates and the cultivated and managed areas, where land is basically used for agricultural purposes, may be attributed to wild boar invading such areas searching for food. The human activity in combination with the presence of domestic pig farms in these areas creates suitable circumstances for the spreading of the PRRS virus to wild boar as the virus can be transmitted not only via direct but also via indirect routes such as vehicles, people involved in the swine industry and flying insects.

The recent rapid demographic expansion of wild boar (*Sus scrofa*) all over Europe, including Greece, may influence the epidemiology of various pathogens that can affect not only livestock but also humans and are transmitted and spread by various routes. Based on hunting bag estimates, the number of wild boar in Greece is increasing year by year and during the last hunting season it exceed 25,000 individuals. Although limited, the evidence of exposure of wild boar to IA virus, *Salmonella* species, *Trichinella* species and *T. gondii*, which are some of the most important zoonotic pathogens worldwide, indicates that this animal species may be important for their

spread and their maintenance in the environment and may represent a health risk for people handling or consuming them. In addition, the unclear situation of the wild boar's infectious status in bordering countries and the possible spread of infectious agents because of their migration across Greek borders makes the regular monitoring of wild boar diseases essential. Furthermore, the role of wild boar as a source of infectious agents for other species should be extensively studied to determine their impact on swine industry.

Table 1: Seroprevalence of 10 selected pathogens in European wild boar in Greece

Pathogen	**Serological assay**	**Seropositivity(percentage; 95% CI)**			
		Overall (n=94)	Mountain range A RODOPI (n=43)	Mountain range B CENTAL (n=28)	Mountain range C PINDOS (n=23)
Porcine circovirus-2 (PCV-2)	ELISA (Ingenasa)	18/94 (19.1%; 11.15-27.05)	5/43 (11.6%; 2.03 - 21.17)	9/28(32.1%; 14.81- 9.39)	4/23(17.4%; 1.91 - 32.89)
Porcine reproductive and respiratory syndrome virus (PRRSV)	ELISA (IDEXX laboratories)	12/94(12.8%; 6.05-19.55)	3/43 (7%; -0.63 - 14.63)	9/28 (32.1%; 14.81 - 49.39)	0/23 (0%)
Aujeszky's disease virus (ADV)	ELISA (IDEXX laboratories)	33/94 (35.1%; 25.45-44.75)	16/43 (37.2%; 22.75 -51.65)	14/28 (50%; 31.48 - 68.52)	3/23 (13%; -0.74 - 26.74)
Influenza A (IA) virus	ELISA (Ingenasa)	1/94 (1.1%;-1.01-3.21)	1/43 (2.3%; -2.18 -6.78)	0/28 (0%)	0/23 (0%)
Actinobacillus pleuropneumoniae	ELISA (IDEXX laboratories)	54/94 (57.4%; 47.4-67.4)	22/43 (51.2%; 36.26 - 66.14)	18/28 (64.3%; 46.55 - 82.05)	14/23 (60.9%; 40.96 - 80.84)
Mycoplasma hyopneumoniae	ELISA (IDEXX laboratories)	0/94 (0%)	0/43 (0%)	0/28 (0%)	0/23 (0%)
***Salmonella* spp**	ELISA (IDEXX laboratories)	4/94 (4.3%; 0.2-8.4)	1/43 (2.3%; -2.18 -6.78)	1/28 (3.6%; -3.3 - 10.5)	2/23 (8.7%; -2.82 - 20.22)
***Trichinella* spp**	ELISA (Institut Pourquier)	6/94 (6.4%; 1.45-11.35)	2/43 (4.6%; -1.66 - 10.86)	1/28 (3.6%; -3.3 - 10.5)	3/23 (13%; -0.74 - 26.74)
Toxoplasma gondii	IFAT (Fuller laboratories)	5/94 (5.2%; 0.71-9.69)	4/43 (9.3%; 0.62 - 17.98)	0/23 (0%)	1/23 (4.3%; -3.99 - 12.59)
Neospora caninum	IFAT (Fuller laboratories)	1/94 (1.1%; 1.01-3.21)	0/43 (0%)	1/28 (3.6%; -3.3 - 10.5)	0/23 (0%)

Fig 1: Map of the study area, showing the origin of wild boar samples found seropositive to various viral agents. Wild boar distribution in the area and the presence of free-ranging swine farms are also demonstrated (according to Tsachalidis and Hadjisterkotis 2009, Giannakopoulos 2012)

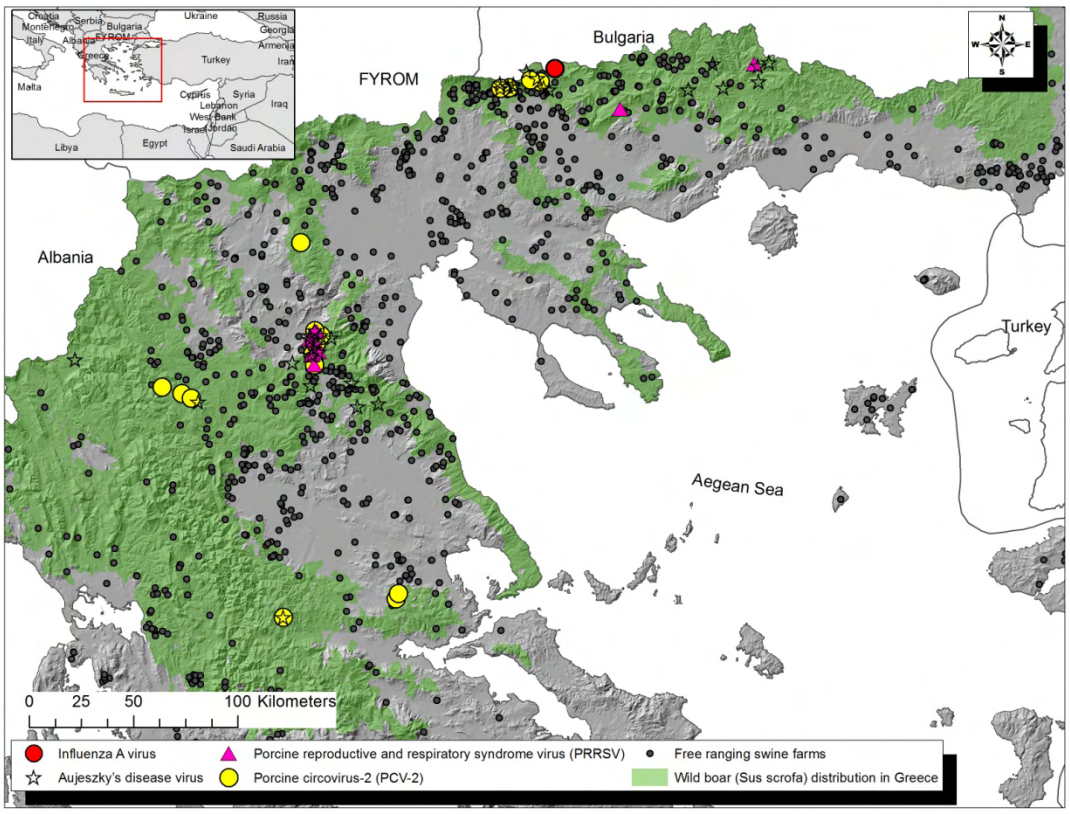


Fig 2: Map of the study area, showing the origin of wild boar samples found seropositive to various bacterial and parasitic agents. Wild boar distribution in the area and the presence of free- ranging swine farms are also demonstrated (according to Tsachalidis and Hadjisterkotis 2009, Giannakopoulos 2012)

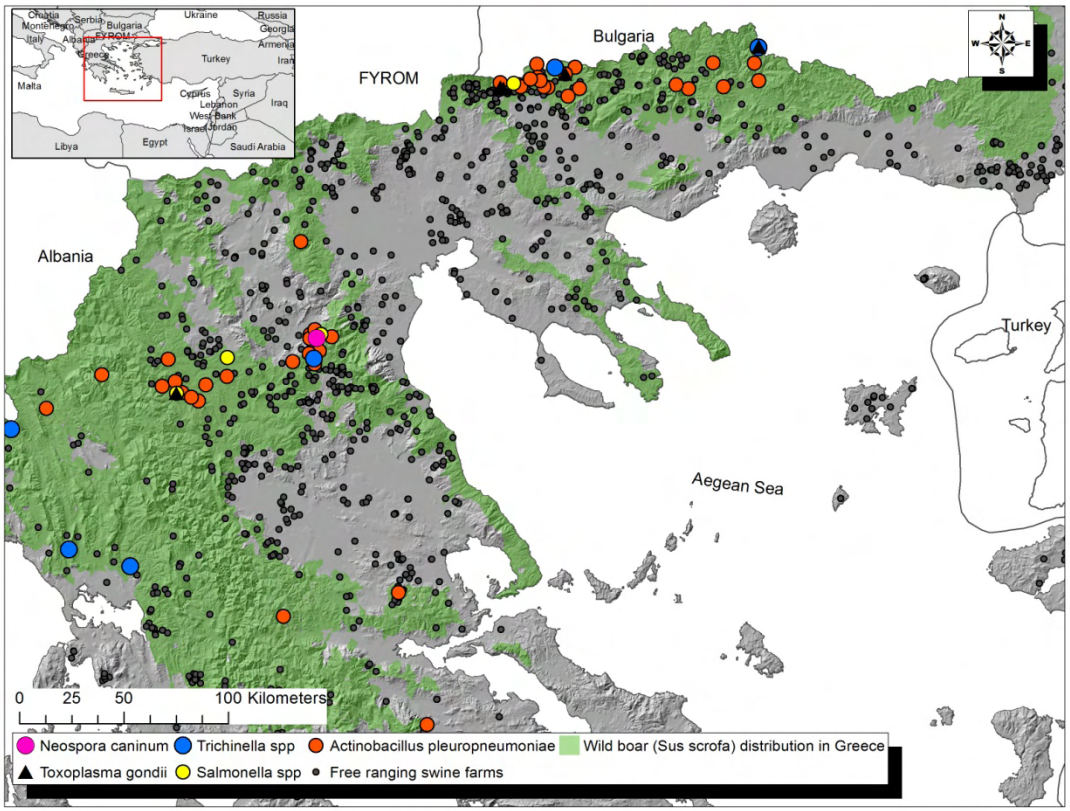


Fig 3: Map of the study area, showing the origin of wild boar samples found seropositive to at least one of the 10 pathogens (red dots) and those found seronegative to all of the 10 pathogens (green dots)

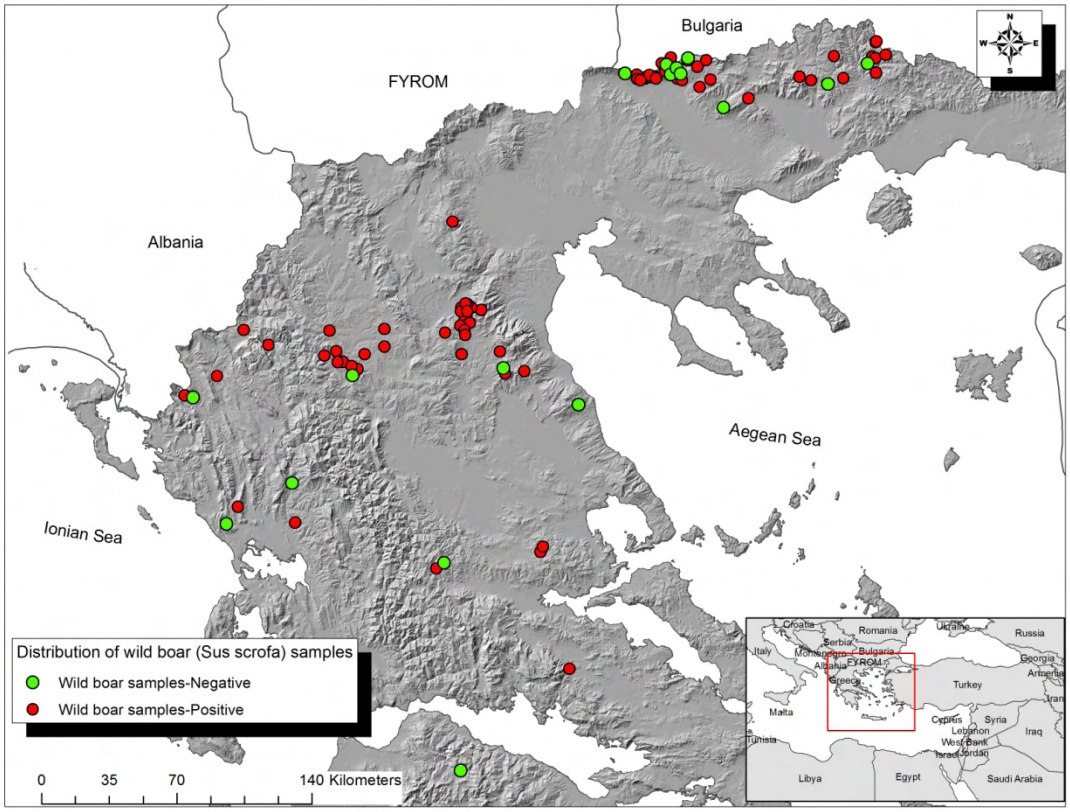
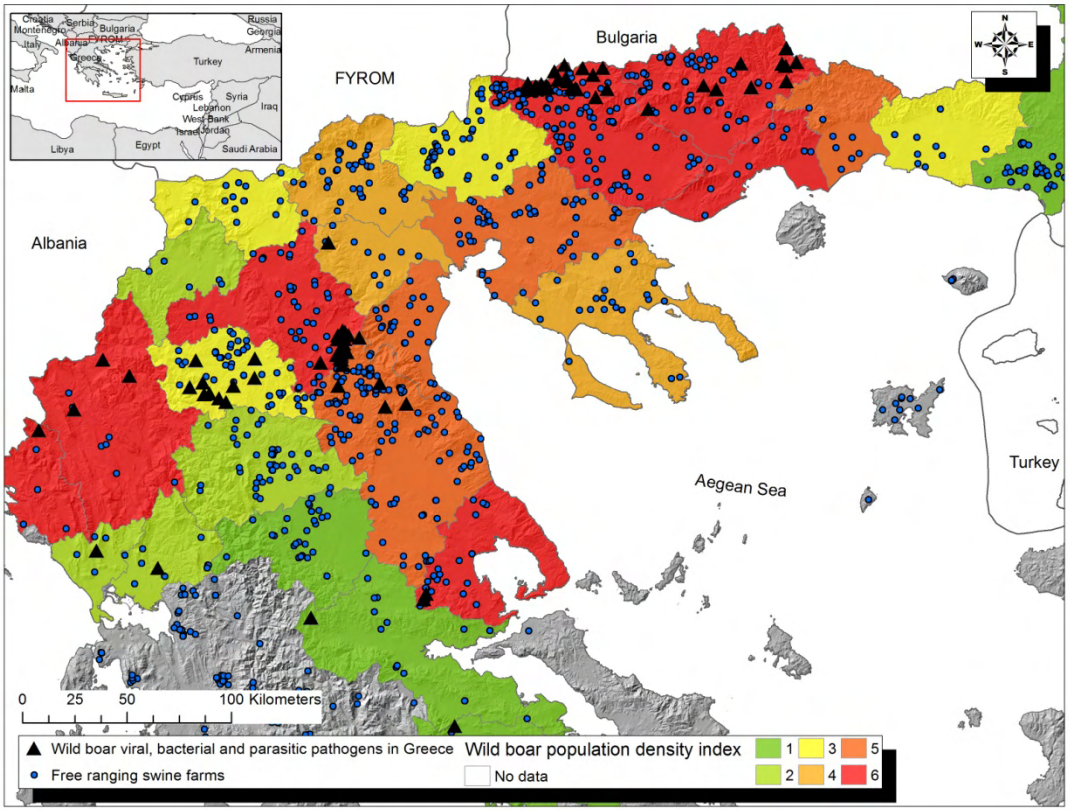
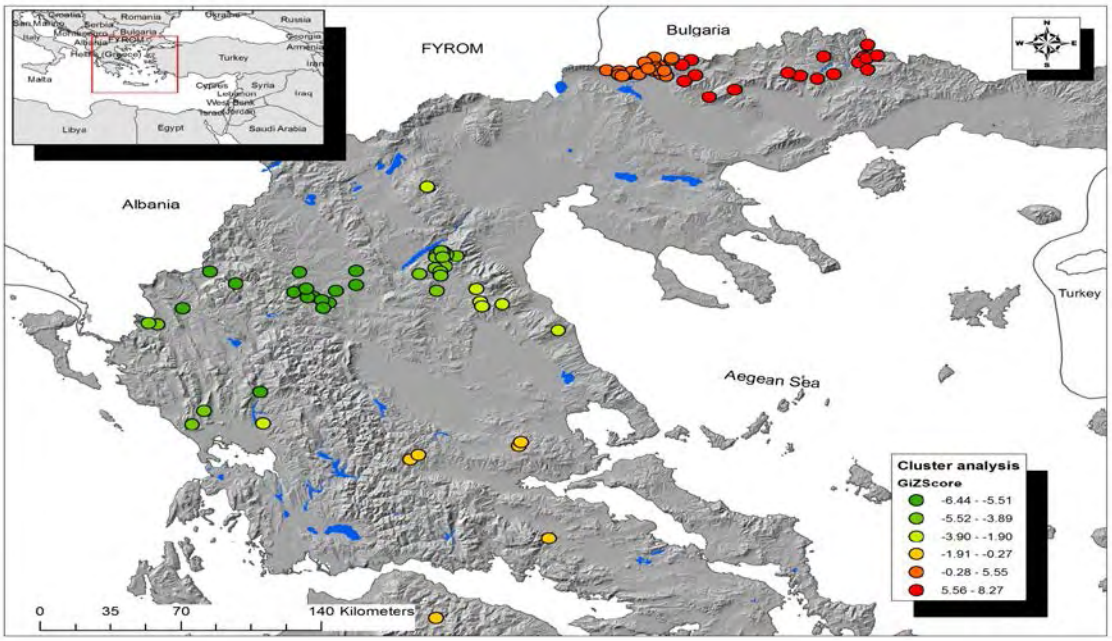


Fig 4: Map of the study area, showing the origin of wild boar samples found seropositive to at least one of the 10 pathogens, the free-ranging swine farms and the overall wild boar population density



Supplementary Figure 1: The Gi* statistic for each feature in the data set appears as a z-score. The latter revealed a cluster (hot spot) of seropositivity to at least one of the pathogens examined near the Bulgarian borders



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Chapter 3: Development of a multiplex bead assay for the detection of serological responses to *Brucella* species that overcomes the cross-reactivity with *Yersinia enterocolitica* O:9 in domestic pig and wild boar population

3.1 Introduction

Porcine brucellosis is a major concern and is widespread throughout the world, especially in the Mediterranean, Balkans, South America and South East Asia. The primary etiologic agent of the disease is the bacterium *Brucella suis*, which is associated with severe economic losses in livestock production and may threaten public health ((OIE, 2014). The Eurasian wild boar (*Sus scrofa*) is widely distributed in the Palearctic and is a wildlife reservoir host for *B. suis* in many regions. Apparent prevalence of *B. suis* (serum antibody prevalence) has been estimated to range from 25-46% in areas of high wild boar density in Spain where epidemiological links with *Brucella* infection in domestic pigs are suspected (Muñoz et al., 2010).

The serological methods that are used for the diagnosis of porcine brucellosis are the indirect, the blocking and the competitive enzyme-linked immunosorbent assays (ELISA) based on smooth lipopolysaccharide antigens (sLPS) the Rose Bengal Test (RBT), the complement fixation test (CFT) and the fluorescence polarization assay (OIE, 2014; Muñoz et al., 2012). The *B. abortus* antigens seem to be appropriate for testing swine sera, at least in RBT and CFT, as they can identify antibodies against the three biovars (1,2,3) of *B. suis*, which cause infection in pigs (OIE, 2014). A drawback of these serological tests is the lack of reliability for individual diagnosis, because, although they may have acceptable sensitivity, they frequently lack specificity (OIE, 2014). A major reason for this is infection by *Yersinia enterocolitica* O:9, due to the closely related structures of *Brucella spp* and *Y. enterocolitica* O:9 sLPS O-chains (Meikle et al., 1989; Cloeckaert et al., 1998; Kittelberger et al., 1998; Jungersen et al., 2006; Nielsen et al., 2006). The structure and the biological properties of the rough *Brucella* LPS make it a suitable antigen for the serological diagnosis of porcine brucellosis. Specifically, it lacks O-chain and thus only the lipid A and the core antigens remain. This rLPS structure is different between *Brucella* and *Y. enterocolitica* O:9 (Kubler-Kielb and Vinogradov, 2013; Barquero-Calvo et al., 2009; Müller-Leonnies et al., 1999). The omission of the cross-reactive O-chain means that rLPS has the potential to be a more specific antigen when applied to samples that are false-positive in assays employing the O-chain (McGiven et al., 2012). In wild boar however, the sensitivity of a multi-

species *Brucella* sLPS iELISA was estimated at 100% and it's specificity was adequate; cross-reactivity with *Y. enterocolitica* O:9 was not assessed (Muñoz et al., 2010).

The above reasons prompted us to use a rLPS rich antigen, extracted from a rough strain of *Brucella*, in order to try to enhance the specificity (Sp) of serology. Additionally, a whole cell *B. suis* 1330 smooth antigen was used to maximize sensitivity (Se) because it is a homologous antigen for infected domestic pigs and wild boars, and being a whole cell antigen it contains most possible immunogenic epitopes, and has a high O-chain content (Cloeckaert, 1990; OIE, 2014). The combination of these two antigens in the same multiplex bead assay reaction could be efficient in enhancing the diagnostic accuracy of the test. Finally, a whole cell *Y. enterocolitica* O:9 antigen was used to detect cross-reacting antibodies and/or antibodies produced after natural exposure to *Yersinia enterocolitica* that may be present in *Brucella* seropositive domestic pigs and wild boar.

The purpose of the present study was to develop a multiplex bead assay using a rLPS, a whole cell *B. suis* 1330 smooth, and a whole cell *Y. enterocolitica* O:9 antigen, that not only discriminates *Brucella* seropositive from *Brucella* seronegative domestic pigs and wild boar but also overcomes the cross reactivity between *B. suis* and *Y. enterocolitica* O:9.

3.2 Materials and methods

3.2.1 Domestic pig sera

One hundred twenty six domestic pig sera were used and allocated into three groups. Group A contained 29 sera from herds that were confirmed by culture [14] to be infected with *B. suis* biovar 2 (25/29) obtained in Spain, or biovar 1 (4/29), obtained in South America. Group B contained 80 randomly selected sera collected from herds within Great Britain, which is officially brucellosis-free. Group C contained 17 sera from herds within Great Britain that presented FPSR (false positive serological

reactors) during routine testing by either RBT (n=10), cELISA (n=10), SAT (n=8) or their combination.

3.2.2 Wild boar sera

Forty nine Eurasian wild boar sera from Spain were used and allocated into two groups. Group A - *Brucella* seropositive, (n=18) and, group B - *Brucella* seronegative (n=31). The discrimination between the seropositive and seronegative wild boar was determined by an indirect ELISA using a sLPS antigen (Muñoz et al., 2010).

3.2.3 Multiplex bead assay

3.2.3.1 Antigen preparation and coupling

The antigens used for the development of the multiplex bead assay included: a) a rLPS rich phenol/chloroform/petroleum ether extract from *B. abortus* RB51 (hereafter referred as rLPS) (McGiven et al., 2012); b) whole cells of the smooth *B. suis* strain 1330 that was grown on serum dextrose agar at 37^{o}C and heat-killed; and c) whole cells of the smooth *Y. enterocolitica* O:9 that was grown on nutrient agar at 27^{o}C and heat-killed. Ten μg of each whole cell antigen, except for rLPS antigen which was diluted to working strength determined by titration to provide optimal diagnostic output, was bound to 2.5 X 10^{6} pro magnetic carboxylated beads according to manufacturer's instruction (Bioplex Pro Magnetic COOH Magnetic Beads Amine Coupling Kit, BioRad).

3.2.3.2 Multiplex bead assay protocol

The following one-step protocol was used after it was validated in terms of repeatability (<10% inter-assay coefficient of variation-CV) and optimization. Specifically, sera were diluted at 1:25, 1:50 and 1:100 in dilution buffer containing 0.1M PBS (pH: 7.2), 1% BSA (w/v) and 0.05% Tween 20 (v/v). Based on the median fluorescence intensity (MFI) values, the 1:50 serum dilution was considered optimal and used for further testing.

Fifty µl of master mix, containing approximately 3,500 coupled beads of each type (the rLPS antigen, the smooth *B.suis* 1330 antigen and the smooth *Y.enterocolitica* O:9 antigen), biotinylated protein AG (secondary antibody) a 1:500 dilution and streptavidin-phycoerythrin (2µg/ml) in dilution buffer, were added to each well of a flat-bottom 96-well plate. Diluted serum (50µl) was mixed with the master mix and the plate incubated for 2h at room temperature, shaking at 600 rpm. The beads were washed twice with 100µl Wash buffer (0.1M PBS and 0.05% Tween 20) using the Bioplex pro Wash Station (BioRad) and finally resuspended in 100µl of dilution buffer. The bead reporter fluorescence, expressed as MFI, was determined using Bioplex 200 (BioRad) instrument. The machine was calibrated and set to count 100 beads from each of three bead sets.

Each sample was tested in duplicate and the average MFI was calculated. On each plate a negative control well containing 50 µl of master mix and 50 µl of dilution buffer was left and used to calculate background MFI. The MFI value of each serum was determined after subtracting the background MFI from the average MFI. Serum from a *Brucella* infected domestic pig was included as a positive-control in each plate and its MFI, after subtracting the background MFI of the plate, was used for normalization of the MFI values of the sera run in the different plates. The MFI normalization was performed by dividing each MFI value of each sample by the MFI value of the corresponding positive-control serum (intra-plate normalization). Additionally, the CV of the MFI values of the positive control serum sample was less than 20% among the different plates (inter-plate variation). Due to the lack of a wild

boar positive-control, the same domestic pig positive-control was also used to normalize wild boar sera MFI values. The normalized MFI values are presented and were used for statistical analysis.

3.2.4 Statistical analysis

Comparison of the normalized MFI values between each of the two *Brucella* spp antigens (rLPS, smooth *B. suis* 1330) with that of the smooth *Y. enterocolitica* O:9 antigen, separately in domestic pig group A and group C was performed using paired t-test, in order to initially evaluate if there is a statistically significant difference between the mean MFI results of the studied populations.

Receiver operating characteristic (ROC) analyses were performed for the following data sets: a) the normalized MFI values for the rLPS and for the smooth *B. suis* 1330 antigen of domestic pig groups A and B; b) the normalized MFI values for the rLPS and for the smooth *B. suis* 1330 antigen of domestic pig groups A and C; c) the ratio between the smooth *B. suis* 1330 and the smooth *Y. enterocolitica* O:9 normalized MFI values of domestic pig groups A and C; and d) the normalized MFI values for the rLPS and for the smooth *B. suis* 1330 antigen of wild boar groups A and B. ROC curves were generated by plotting the true-positive rate (Se) against the false-positive rate (1-Sp). The area under the curve (AUC) was calculated as an overall measure of the discriminatory power of the assays and the cut-off values with the maximum possible sum of Se and Sp - 1 (Youden Index) were calculated (Greiner et al., 2000). Based on the cut-offs of normalized MFI values for the rLPS and for the smooth *B. suis* 1330 antigen of domestic pig groups A and B, the specificity (Sp) of the multiplex bead assay for domestic pig group C was calculated.

Statistical analysis was carried out using SPSS v20.

3.3 Results

The distribution of the MFI values for the two *Brucella* spp antigens and the *Y. enterocolitica* O:9 antigen for all domestic pig sera are shown on the Figure 1. In group A domestic pig the normalized MFI values for rLPS and for smooth *B. suis* 1330 antigens were significantly higher compared to the normalized MFI values for smooth *Y. enterocolitica* O:9 antigen ($P<0.0001$ and $P<0.0001$, respectively) whereas the opposite was true for group C domestic pig ($P<0.0001$ and $P<0.0001$, respectively). In domestic pig group A and B sera, ROC analysis of the normalized MFI values for rLPS antigen showed AUC=0.958 (95% CI: 0.920 - 0.996) and for the smooth *B. suis* 1330 antigen showed AUC=1 (95% CI: 1.000 - 1.000). In domestic pig group A and C sera, ROC analysis of the normalized MFI values for rLPS antigen showed AUC=0.957 (95% CI: 0.904 - 1.000) and for the smooth *B. suis* 1330 antigen showed AUC=0.722 (95% CI: 0.564 - 0.880). The ROC analysis of the ratio between the smooth *B. suis* 1330 and the smooth *Y. enterocolitica* O:9 normalized MFI values showed AUC=1 (95% CI: 1.000 - 1.000); the relevant cut-off values, the Se and Sp of multiplex bead assay are shown on Table 1.

The distribution of the MFI values for the two *Brucella* spp antigens and the *Y. enterocolitica* O:9 antigen for all wild boar sera are shown on the Figure 2. In wild boar group A and B sera, ROC analysis of the normalized MFI values for rLPS antigen showed AUC=0.975 (95% CI: 0.940 - 1.000) and for the smooth *B. suis* 1330 antigen showed AUC=0.993 (95% CI: 0.977 - 1.000); the relevant cut-off values, the Se and Sp of multiplex bead assay are shown on Table 2.

Twenty six of the 29 group A domestic pigs (23/25 *Brucella* infected by biovar 2 and 3/4 infected by biovar 1) were positive using the rough *B. abortus* RB51 antigen, while 75/ 80 group B domestic pigs and all (17/17) group C (FPSR) domestic pigs were negative. The same antigen detected 15/18 of the seropositive wild boar and was negative for all (31/31) seronegative wild boar.

The smooth *B. suis* 1330 antigen detected all (29/29) group A domestic pigs, was negative in all (80/80) group B animals, but it was positive in most (13/17) group

C (FPSR) samples. In wild boar the same antigen detected all (18/18) group A seropositive samples and was negative in 30/31 group B seronegative animals. Finally, the ratio of the smooth *B. suis* 1330 and the smooth *Y. enterocolitica* O:9 normalized MFI values discriminated with 100% sensitivity and 100% specificity group A and group C (FPSR) domestic pigs.

The experiment was repeated three times and the reproducibility of the results (positive/negative) was 100%.

3.4 Discussion

This study shows that the multiplex bead assay could be a useful serodiagnostic tool for porcine brucellosis due to the high Se and Sp (whole cell *B. suis* 1330 smooth antigen) and the ability to identify cross-reactions due to *Y. enterocolitica* O:9 (rLPS antigen; smooth *B. suis* 1330/smooth *Y. enterocolitica* O:9 normalized MFI values ratio).

Most commonly used serological tests are generally designed to measure antibodies against a single antigen preparation (which may be one or more individual antigens), whereas the one-step multiplex bead assay is capable of detecting individual antibody responses to a range of different antigens at the same time. This offers significant benefits over other tests, including reduced reagent costs (Pickering et al., 2002). The potential advantages of multiplex bead assays over conventional serologic tests provide a strong impetus for their routine use in both research and clinical laboratories. In our study the beads were conjugated on 3 separate occasions and were easily mixed and efficiently combined.

The frequency of the false-positive reactions in group B domestic pigs and wild boar was low (< 10%), despite the fact that multiplexed assays are usually characterized by lower Sp compared to conventional serologic tests due to simultaneous presence of multiple ligands (Elshal et al., 2006). One possible explanation could be the mixture of all reagents in one step and the use of protein AG

instead of a species/isotype-specific secondary antibody. The results of this study show that the multiplex bead assay using rLPS and smooth *B. suis* 1330 antigens effectively discriminates sera from group A and group B in both domestic pigs and wild boar. Based on the calculated AUC values, Se and Sp the latter antigen seems to be better than rLPS for this purpose.

In a recent study, the Se and Sp of a conventional iELISA, in which sLPS antigen was used, were 66.3% and 96.9%, respectively, leading to the conclusion that this assay is not sensitive enough for the diagnosis of brucellosis in domestic pigs (Praud et al., 2012). In another study, the use of sLPS antigen in the iELISA resulted in 94.1% Se and 100% Sp (McGiven et al., 2012), confirming the results of several previous publications that also support the diagnostic accuracy of sLPS in discriminating *Brucella*-infected from non-infected domestic pigs (EFSA, 2009; Paulo et al., 2000). Our results indicate that a multiplex bead assay may be better than conventional serologic tests to discriminate sera from *Brucella* infected and non-infected non-FPSR domestic pigs using the whole smooth *B. suis* 1330 antigen (Se:100%, Sp:100%).

The use of the rLPS had a satisfactory diagnostic performance in the multiplex bead assay. This good distinction between the group A and group B samples from domestic pigs was also found in a recent study, where the same antigen was used in an iELISA, resulted in a Se of 91.2% and a Sp of 98.8% (McGiven et al., 2012).

The application of serological tests in wildlife is usually carried out for screening purposes or surveillance. Wild boar are indigenous in many countries and may contribute to the transmission of *B. suis* to livestock and hamper the success of eradication programs (Godfroid et al., 2002). Based on our results, the antigen which fits better for screening purposes in wild boar population using the multiplex bead assay is the smooth *B. suis* 1330 (Se: 100%, Sp 96.8%). Furthermore, given the high seroprevalence (up to 63%) against *Brucella* spp in European wild boar population (Grégoire et al., 2012), the concomitant use of rLPS may be advantageous to improve the combined specificity, considering that this antigen gave negative results in one of the two group B sera with positive normalized MFI values for the smooth *B. suis* 1330 antigen (data not shown). Further studies with larger sample sizes are obviously needed to confirm this hypothesis.

In our study the cut-offs were based on the maximum sum of Se and Sp. According to Greiner et al. (2000), the cut-off should be selected taking into consideration the epidemiologic situation in each area. For example, for countries which are brucellosis-free, taking into account the low prevalence of the disease and the serious consequences of a false positive diagnosis, it may be advisable to choose a cut-off at the lower part of the ROC curve in order to maximize the Sp. On the other hand, maximum Se would be appropriate for countries where the disease occurs at high prevalence. Therefore, the Se and Sp of the multiplex bead assay may change if different criteria for the selection of the cut-off become adopted.

The results of the multiplex bead assay clearly show that in domestic pig the rLPS antigen can discriminate group A (sera from *Brucella* infected animals) from group C (sera from non-*Brucella* infected FPSR samples). This attribute of the rLPS rich antigen was expected, as the structure of the rLPS is very different between *Brucella* spp (Velasco et al., 2000) and *Y. enterocolitica* O:9 (Muller et al., 1999) and the negligible amount or even absence of the O-chain in this antigen (Cloeckaert et al., 2002) does not permit cross-reactivity with antibodies against *Y. enterocolitica* O:9, as has been previously shown using an iELISA method (McGiven et al., 2012).

The inability of the smooth *B. suis* 1330 antigen to differentiate between group A and group C domestic pigs was also expected, based on previous studies in cattle (Emmerzaalv et al., 2002; Munoz et al., 2005) and pigs (Jungersen et al., 2006; Nielsen et al., 2006). However, the concomitant use of the smooth *Y. enterocolitica* O:9 antigen in the multiplex bead assay and the calculation of the ratio between the smooth *B. suis* 1330 and the smooth *Y. enterocolitica* O:9 normalized MFI values fully overcame this drawback permitting the clear differentiation between groups A and C. Additionally, the use of the rLPS antigen in a multiplex bead assay may be helpful in cases of dual infections of *Brucella* spp and *Y.enterocolitica* O:9, given that the OPS is so similar between the *B. suis* biovar 2 and the *Y.enterocolitica* O:9 (Zaccheus et al., 2013)

Based on the results of this study multiplex bead assay can be considered an accurate diagnostic test for brucellosis in domestic pigs and wild boar, if at least two antigens are included. For domestic pigs, the use of the smooth *B. suis* 1330 antigen along with the *Y. enterocolitica* O:9 antigen (thus permitting to calculate the ratio

between MFI values of these two antigens) seems to be the best combination to discriminate sera from *Brucella* infected and non-*Brucella* infected (FPSR and non-FPSR). In wild boar the smooth *B. suis* 1330 antigen seems to be more accurate in terms of Se and Sp but the addition of the rLPS may further increase Sp.

Table 1. Diagnostic performance of the multiplex bead assay for the domestic pigs groups. Diagnostic performance of the multiplex bead assay for the two *Brucella* antigens and for the ratio between the smooth *B. suis* 1330 and the smooth *Y. enterocolitica* O:9 normalized MFI values in the *Brucella* infected (Group A) domestic pigs, in the non-*Brucella* infected false positive serological reactors (Group C) and in the non-*Brucella* infected domestic pigs (Group B).

		Antigen and cut-off (in parenthesis)		
		B. suis 1330 (3,90%)	RB51 rLPS (3,96%)	*B.suis* 1330/*Ye* O:9 (12,69%)
Groups	Parameters			
A vs B	ROC AUC[1] (95% CI)	1.000 (1.000 - 1.000)	0.958 (0.921 - 0.996)	N/A
	Se[2] (95% CI)	100 (87.9 - 100)	89.7 (72.6 - 97.7)	N/A
	TP/P[3]	29/29	26/29	
	Sp[4] (95% CI)	100 (95.5 - 100)	93.8 (86.0 - 97.9)	N/A
	TN/N[5]	80/80	75/80	
A vs C	ROC AUC (95% CI)	0.722 (0.564 - 0.880)	0.957 (0.904 - 1.000)	1.000 (1.000 - 1.000)
	Se (95% CI)	100 (87.9 - 100)	89.7 (72.6 - 97.7)	100 (87.9 - 100)
	TP/P	29/29	26/29	29/29
	Sp (95% CI)	23.5 (7.0 - 49.9)	100 (80.3- 100)	100 (80.3- 100)
	TN/N	4/17	17/17	17/17

[1] AUC: area under the curve; [2]Se: sensitivity; [3]TP/P: True Positive/Positive; [4]Sp: specificity; [5]TN/N: True Negative/Negative

Table 2. Diagnostic performance of the multiplex bead assay for the wild boar groups Diagnostic performance of the multiplex bead assay for the two *Brucella* antigens in the *Brucella* -seropositive wild boar (Group A) and in the *Brucella*- seronegative wild boar (Group B).

		Antigen and cut-off (in parenthesis)	
		B. suis 1330 (4,66%)	RB51 rLPS (5,61%)
Groups	Parameter		
A vs B	ROC AUC[1] (95% CI)	0.993 (0.977 - 1.000)	0.975 (0.940 - 1.000)
	Se[2] (95% CI)	100 (81.3 - 100)	83.3 (58.6 – 96.2)
	TP/P[3]	18/18	15/18
	Sp[4] (95% CI)	96.8 (83.2 - 99.5)	100 (88.7 - 100)
	TN/N[5]	30/31	31/31

[1] AUC: area under the curve; [2]Se: sensitivity; [3]TP/P: True Positive/Positive; [4]Sp: specificity; [5]TN/N: True Negative/Negative

Fig 1: Distribution of the median fluorescence intensity (MFI) values for the two *Brucella* spp antigens and the *Y. enterocolitica* O:9 antigen in the *Brucella* infected domestic pigs (Group A), the non-*Brucella* infected domestic pigs (Group B) and the *Brucella* false positive serological reactors domestic pigs (Group C). The dots represent the outliers and the asterisks represent the extreme outliers.

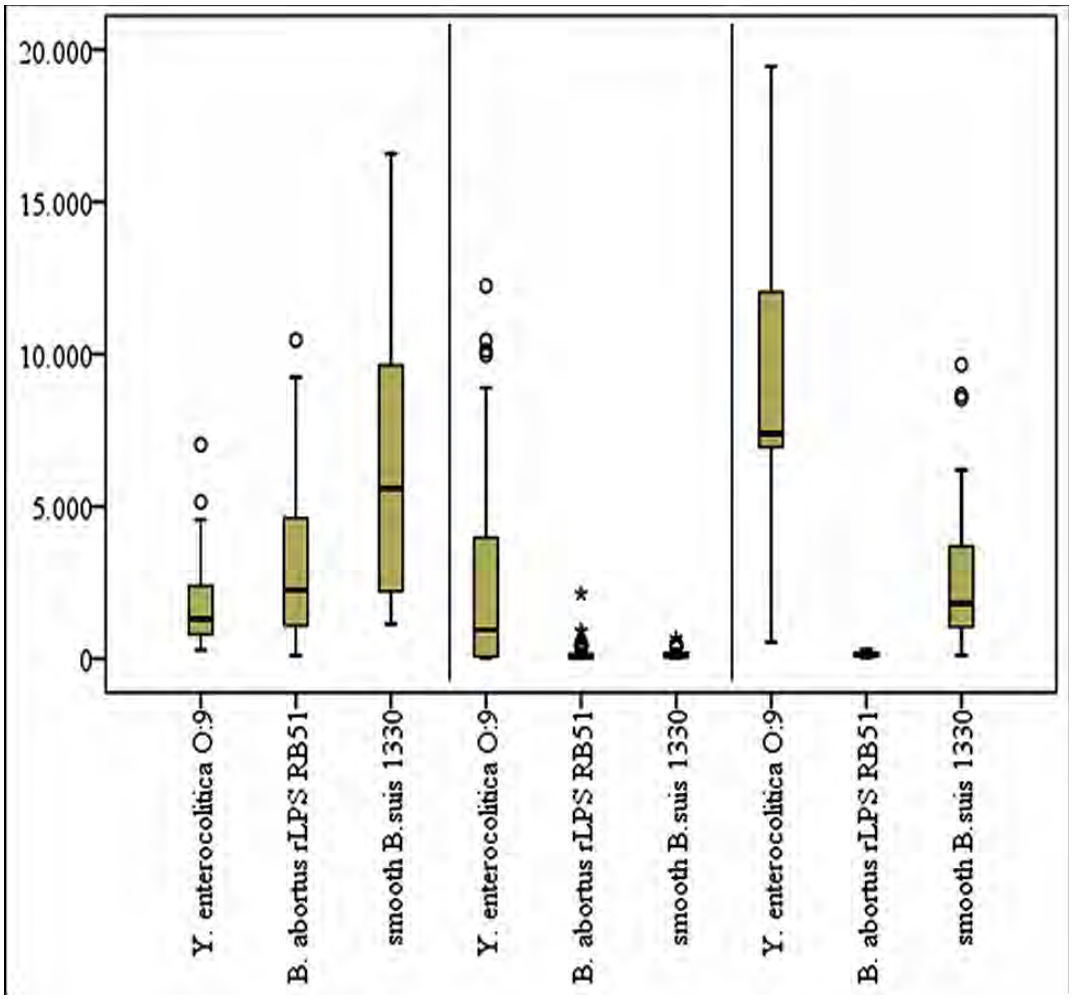
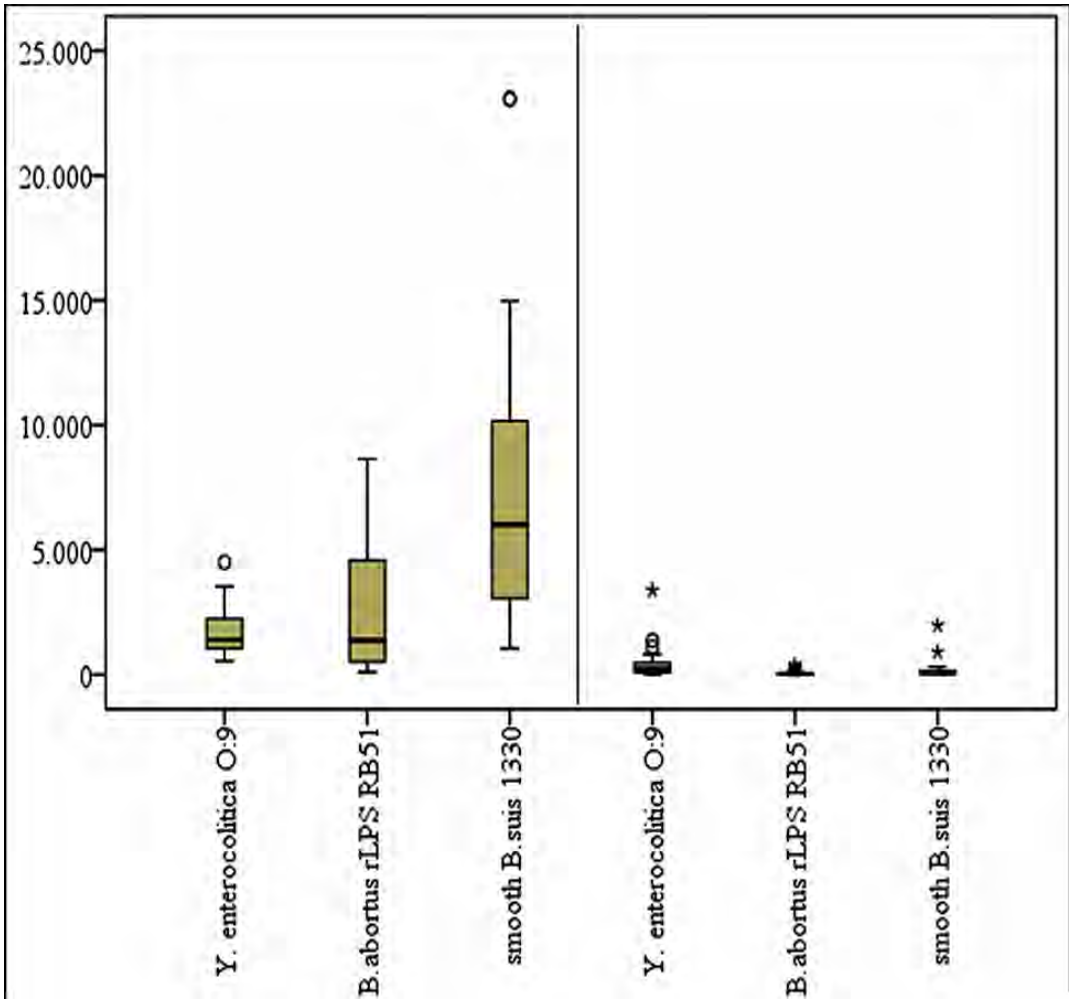


Fig 2: Distribution of the median fluorescence intensity (MFI) values for the two Brucella spp antigens and the Y. enterocolitica O:9 antigen in the Brucella seropositve wild boar (Group A) and for Brucella seronegative wild boar (Group B). The dots represent the outliers and the asterisks represent the extreme outliers.



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Chapter 4: Diagnostic performance of a multiplex bead assay and a serology microarray technology as serodiagnostic tools for the simultaneously detection of antibodies against *Mycobacterium bovis*, *Brucella suis* and *Trichinella spiralis* in wild boar

4.1 Introduction

Mycobacterium bovis, *Brucella suis* and *Trichinella spiralis* are potential pathogens for the domestic animals and humans, and wild boar can be an important source for their maintenance and transmission among species (Meng et al., 2009). Same strains of *M. bovis*, the causative agent of bovine tuberculosis, have been isolated from wild boar and cattle (Serraino et al., 1999; Zanella et al., 2008), leading to speculation that the former may not be a dead-end host but a true wildlife reservoir of the bacterium. In addition, humans can become infected through consumption of contaminated wild boar products and direct contact with wounds of infected animals, which is especially important for hunters and veterinarians (CDC). Although biovars 1 and 3 are the most common biovars of *B. suis* that affect wild boar, domestic pigs and humans, biovar 2 may also be transmitted, under certain circumstances, from wild boar to domestic pigs (Cvetnic et al. 2003) and rarely to humans (Paton et al., 2001, OIE, 2009). Finally, wild boar play a key role in the maintenance and spread of *T. spiralis* and in regions where their population density is low and eradication programs against the parasite are into effect, the infection is rare (Pozio, 2007). Specifically, these species are the most important source of infection for domestic pigs, which in turn are the main source of infection for other domestic animals and humans (Pozio and Muller, 2006).

The most widely used method for bovine tuberculosis diagnosis is the tuberculin skin test using purified protein derivative that causes a delayed-type hypersensitivity reaction in infected animals. The main disadvantages of this test are the low specificity (Pollock et al., 2005) and the inability to perform confirmatory tests for the next 2-3 months. Consequently, this test is not only impractical for wild animal species, but also the long delay until implementation of confirmatory tests increases the risk of disease spreading (Lilenbaum et al., 1999). Therefore serological assays for detection of early antibody responses to *M. bovis* in wild boar are necessary.

Many serological tests are available for the diagnosis of porcine brucellosis, such as the indirect, blocking and competitive enzyme-linked immunosorbent assays (ELISA) based on smooth lipopolysaccharide antigens (sLPS), the Rose Bengal Test

(RBT), the complement fixation test (CFT) and the fluorescence polarization assay (OIE, 2009), but none of them is commercially available for wild boar.

The systematic tracking of trichinellosis in swine and wild boar is based on the direct detection of the larvae, either by trichinoscopy or by muscle digestion, which is considered the most reliable post-mortem diagnostic method. There are also commercial ELISAs available for detection of antibodies against *T. spiralis* in serum and meat juice of swine, wild boar and horses. They are typically based on the use of excretory-secretory (E/S) antigens, which are metabolic products of larvae.

A multiplexed assay for the simultaneous detection of antibodies against these pathogens in wild boar would be a useful screening tool. The aim of the study was to develop and evaluate a multiplex bead assay for the simultaneous detection of antibodies against *M. bovis*, *B. suis* and *T. spiralis* in wild boar sera, and to compare it's performance with a printed antigen microarray (array ref) and conventional single-pathogen ELISAs. The multiplex bead assay was based on BioRad Bio-Plex system of a multi-analyte suspension array which is based on Luminex's XMAP technology. The serology microarray was based on the ArrayStrip (AS) platform by Alere Technologies GmBH.

4.2 Materials and methods

4.2.1 Serum samples

Sera from Eurasian wild boar were used for the evaluation of the two multiplex assays. For the TB assays, 64 seropositive and 105 negative animals from Spain were tested. For *Brucella*, 29 seropositive and 39 negative Spanish boar were used. The serological statuses of these animals were determined previously using validated ELISAs (TB - Boadella et al. 2011; *Brucella* - Muñoz et al., 2010)., Further, 21 seropositive and 97 negative sera of wild boar from Spain and Greece were used for the development and the evaluation of serological assays for trichinellosis. These sera had been tested by a

commercial ELISA (IDEXX Trichinellosis Ab Test) for the detection of antibodies against *T. spiralis*, as a gold standard test.

4.2.2 Multiplex bead assay

The BioRad Bio-Plex multi-analyte bead suspension array system, which is based on Luminex's XMAP Technology, was used for the assay development. The antigens used were: a) recombinant MPB83 antigen (Lionex, Germany) for the detection of antibodies against *M. bovis* b) an in-house, whole-cell preparation of the smooth *B. suis* 1330, that was grown on serum dextrose agar at 37oC and heat-killed, and c) an in-house, *T. spiralis* 3rd instar larval crude antigen preparation. Each antigen was coupled to differently marked Bio-Plex Pro Magnetic COOH beads (10 µg/2.5 x 10^6 beads) using an Amine Coupling Kit according to manufacturer's instruction (BioRad).

The following one-step protocol was used after it was validated in terms of repeatability (<10% inter-assay coefficient of variation-CV) and optimization (results not shown). Based on the median fluorescence intensity (MFI) values of a range of serum dilutions, a 1/50 dilution was considered optimal (data not shown) and used for further testing.

A total of 50µl of master mix, containing approximately 3,500 coupled beads of each of the three bead sets, biotinylated protein AG (Thermo Scientific) at 1:500 dilution (0.1 µl per well) and 2µg/ml streptavidin-phycoerythrin. in dilution buffer containing 0.1M PBS (pH: 7.2), 1% BSA (w/v) and 0.05% Tween 20 (v/v)., were added to each well of a flat-bottom 96-well plate. Fifty µl serum, (diluted 1/25) were then added (giving a final dilution of 1/50) and incubated for 2h at room temperature on a shaker, set at 600 rpm. Beads were then washed twice with 100µl Wash buffer (0.1M PBS and 0.05% Tween 20) using the Bio-Plex pro Wash Station (BioRad) and finally resuspended in 100µl of dilution buffer. The bead reporter fluorescence, expressed as MFI, was determined with a Bioplex 200 (BioRad) instrument that was initially calibrated and set to count 100 beads from each of three bead sets, with the DD Gate values set at 7500-25000.

Each sample was tested in duplicate and the average MFI calculated. On each plate a negative control well containing 50 μl of master mix and 50 μl of dilution buffer was included and used to calculate background MFI. The MFI value of each serum was determined by subtracting the background MFI from the average MFI. A positive-control serum sample of each pathogen (*M. bovis*, *B. suis* and *T. spiralis*) from experimentally infected domestic pigs that were confirmed as positive using standard diagnostic methods, was included in each plate and its MFI, after subtracting the background MFI of the plate, was used for normalization of the MFI values of the sera run in the different plates. Specifically the MFI normalization was performed by dividing the MFI value of each sample by the MFI value of the corresponding positive-control serum (intra-plate normalization). Additionally, the CV of the MFI values of the positive control serum sample was <20% among the different plates (inter-plate variation). The normalized MFI values were used for statistical analysis.

4.2.3 Antigen microarray

A microarray chip was developed to screen wildlife sera for antibodies against multiple pathogens, including the recombinant MPB83, whole cell *B. suis* 1330 and the *T. spiralis* antigens. Wild boar sera were tested on the array at 1/50 dilution as described previously (Cawthraw at al., unpublished data). For the evaluation of the microarray, positive control serum samples for each pathogen were used in each plate. The average signal of the three spots of each antigen was calculated for each sample and the following normalization procedure was applied: a) the local background (the higher of the two negative internal controls) was subtracted from the signal of the antigen to calculate the net signal, b) the ratio (S) of the net signal of the antigen to the net signal of the corresponding positive control was calculated, c) the normalized signal (A) was calculated as $A= (S/\mu)/R$, where μ: the mean of S for all the sera of each pathogen, R: the average signal of the internal positive control.

4.2.4 Conventional single-pathogen ELISAs

We performed two conventional one-pathogen ELISAs, one using recombinant MPB83 antigen and another using the whole cell *B. suis* 1330 antigen. Each antigen was diluted

(1μg/ml and 5μg/ml, respectively) in bicarbonate coating buffer (Sigma) and 100μl were added to each well of a flat-bottom 96-well microtitre plate (Polysorb, Nunc) and incubated overnight at room temperature. Plates were washed 3 times with ELISA wash buffer (0.1 M PBS pH 7.2, 0.05% (v/v) Tween-20) and then blocked with dried skimmed milk (200 μl at 3% w/v, incubated for 30 min at 37^{o} C). After washing, 100 μl of diluted sera (1/50 which was the optimal dilution; data not shown) including a positive control, were added. The plates were incubated for 1 h at 37^{o} C, washed, horseradish peroxidase-conjugated protein A/G (1:10,000) was added and the plates were incubated at 37o C for 30 min. After washing, 100 μl of TMB substrate were added and the plates incubated at room temperature, in the dark for 10 min., The reaction was stopped by adding 50 μl of 10% (v/v) H_2SO_4 and the absorbencies at 450nm (A_{450}) read on a spectrophotometer.

The normalization of the ELISA A_{450} values of the test sera was achieved by dividing the test sample A_{450} values with that of the positive-control (intra-plate normalization). The CV of the A_{450} values of the positive control serum sample were less than 10% between plates (inter-plate normalization) for TB and *Brucella* ELISAs.

4.2.5 Statistical analysis

Receiver operating characteristic (ROC) analyses were performed for the following data sets: a) the normalized MFI values of the recombinant protein of MPB83 antigen, b) the normalized array signals of the recombinant protein of MPB83 antigen, c) the normalized ELISA OD values of the recombinant protein of MPB83 antigen, d) the normalized MFI values of the whole cell *B.suis* 1330 antigen, e) the normalized array signals for the whole cell *B.suis* 1330 antigen, f)) the normalized ELISA OD values of the whole cell *B.suis* 1330 antigen, e) the normalized MFI values of the E/S *T.spiralis* antigen, f) the normalized array signals of the E/S *T.spiralis* antigen. ROC curves were generated by plotting the true-positive rate (Se) against the false-positive rate (1-Sp). The area under the curve (AUC) was calculated as an overall measure of the

discriminatory power of the assays and the cut-off values were determined using the Youden index (Se+Sp-1).

The results for each antigen (MPB83, *B. suis* 1330 and E/S *T. spiralis*) were compared among the serological assays (bead assay, microarray, conventional one-pathogen ELISA) as well as between each serological assay and the gold standard using the Mc Nemar's test.

Statistical analysis was carried out using SPSS v20 and significance was evaluated at the 95% level.

4.3 Results

For wild boar sera of known TB serological status, ROC analysis of the normalized MFI values of multiplex bead assay showed AUC=0.948 (95% CI: 0.915-0.981), ROC analysis of the normalized microarray signals showed AUC=0.962 (95%CI: 0.930-0.994) and ROC analysis of the normalized OD values of the conventional ELISA showed AUC=0.933 (95% CI: 0.895-0.972). The cut-off values, Se, Sp, positive likelihood ratio (PLR), and negative likelihood ratio (NLR) of all the assays are shown on Table 1.

For sera of known *Brucella* serological status, ROC analysis of the normalized MFI values of the multiplex bead assay showed AUC=0.997 (95%CI: 0.991-1), ROC analysis of the normalized microarray signals showed AUC=0.974 (95%CI: 0.941-1) and ROC analysis of the normalized OD values of the conventional ELISA showed AUC= 1 (95% CI: 1-1). The cut off values, Se, Sp, PLR, and NLR of all the assays are shown on Table 2.

For sera of known *T. spiralis* serological status wild boar, ROC analysis of the normalized MFI values of the multiplex bead assay showed AUC=0.973 (95%CI: 0.937-1) and ROC analysis of the normalized microarray signals showed AUC=0.893 (95% CI: 0.748-1).The cut off values, Se, Sp, PLR, and NLR of the two assays are shown on Table 3.

Pair-to-pair comparison of rec.MPB83, results from the gold standard and the multiplex bead assays showed a significant difference (P=0.001), as the multiplex bead assay was less specific, whereas differences between the gold standard and microarray, and between the gold standard and the conventional TB ELISA were not significantly different (P=0.180 and P=0.096, respectively).

Pair-to-pair comparison of the *B. suis* 1330 antigen results from the gold standard and the bead assay, microarray or conventional *Brucella* ELISA showed no significant differences (P=1, P=0.250 and P=1, respectively).

Pair-to-pair comparison of the E/S *T. spiralis* antigen results from the commercial ELISA and the multiplex bead assay and microarrays showed no significant differences (P=1 for both comparisons).

The comparison of the results among multiplex bead assay, microarray technology and conventional ELISAs for each antigen is shown on Table 4.

4.4 Discussion

This study shows the diagnostic potential of the multiplexed assays for the simultaneous detection of antibodies against *M. bovis*, *B. suis* and *T. spiralis* in wild boar. Multiplex bead assay seems preferable to the microarray technology since the latter has the disadvantage that many sera had to be excluded because of the high background noise.

Although studies in cattle propose multi-antigen assays for the diagnosis of bovine tuberculosis in order to increase sensitivity (Coad et al., 2010), the risk of cross-reactivity due to immunoglobulins directed against shared antigens expressed by other bacteria is increased. The relatively early appearance of anti-MPB83 antibodies, around 4 weeks post-infection in cattle (Waters et al., 2006) and the increased Se of serology using MPB83 antigen in experimentally infected cattle and goats (Waters et al., 2006; de Val Pérez et al., 2011) were the reasons for the selection of this single antigen for our assays.

Disregarding the large number of invalid serum samples, the microarray technology using the recombinant MPB83 antigen appeared to be more specific compared to the two other serological assays and its results did not differ significantly from those of the gold standard. Although the results of the multiplexed bead assay differ significantly from the gold standard, as the first is less specific, the high sensitivity (98.4%) makes it a useful serodiagnostic tool for the detection of antibodies against *M. bovis*. In comparison, the conventional ELISA was less sensitive (92% vs 98%) but slightly more specific (86% vs 88%), and did not differ significantly from gold standard test so it may be a useful screening test.

A number of antigens have been used for the development of serological assays for the detection of antibodies against *M. bovis* in wild boar population, giving results similar to that obtained with the three assays tested in the present study (Aurtenetxe et al, 2008).However, the MPB83 antigen showed better diagnostic performance in an conventional ELISA performed in wild boar sera than in the present study, maybe due to differences in the final concentrations of the antigen (0.5mg/ml) and the secondary antibody used (García-Bocanegra et al., 2012).

The whole cell *B. suis* antigen used in the multiplex bead assay and the conventional ELISA was selected because it contains a broad range of antigens and epitopes, and because of its' high O-chain content (Cloechaert, 1990, OIE, 2009). The results of all three serological assays did not differ significantly from each other or with the results of gold standard. The conventional ELISA discriminated efficiently between *Brucella* seropositive and negative boars (Se & Sp = 100%). The multiplexed bead assay and array gave similar results (Se/Sp = 100%/97.4% and 100%/91.7% respectively).

The disadvantage of almost all the serological methods using smooth *Brucella* antigens is that they lack specificity (OIE, 2009; Olsen, 2010), because of the closely related structures of *Brucella* spp and *Y. enterocolitica* O:9 sLPS O-chains. However, the good diagnostic performance of the smooth antigens in discriminating *Brucella* infected from non-infected domestic pigs has been confirmed by several publications (Paulo et al., 2000; EFSA, 2009; Mc Given et al., 2012).

The selection of an E/S antigen in serological assays to differentiate *Trichinella* seropositive from seronegative samples was based on its good diagnostic performance in different serological assays, showing Se of 93.1-99.2% and Sp of 90.6-99.4% (Murrell et al., 1986; Van der Leek et al., 1992; Bień, 2006). These satisfactory results indicate that serology based on E/S antigens may be a useful screening test for trichinellosis for both swine and wild boar (Korínková et al., 2008; Møller et al., 2005). In our study, the use of the E/S *Trichinella* antigen in the multiplex bead assay and serology microarray technology, resulted in high Se and Sp and the results did not differ from those of the commercial ELISA, showing usefulness of these multiplexed assays as screening tools for the detection of antibodies against *T. spiralis* in wild boar.

The numerically (not significantly) lower Sp values of both multiplexed assays in comparison to the conventional single-antigen ELISAs were expected, because as the number of antigens that are used in a serological assay increases, the Sp decreases (Elshal et al., 2006). However, sensitivities and specificities of all three antigens in these assays are still good enough to suggest they have merit for screening purposes at least. Both tests (bead and microarray) become more valuable when one takes into account their multiplex nature which allows screening for exposure to multiple pathogens in a single process. Furthermore, the use of conjugated protein A/G enables these assays to be used to test simultaneously multiple mammal species. These properties are particularly important for wildlife surveillance as samples are frequently in limited supply and there may be no species-specific reagents available.

Table 1. Diagnostic performance of multiplex bead assay, microarray and conventional ELISA for *M. bovis* (recombinant MPB83 antigen) in wild boar sera using a validated ELISA as the gold standard

	Multiplex bead assay	Microarray	Conventional ELISA
Sera	64 positive, 105 negative	25 positive, 92 negative	64 positive, 105 negative
Cut-off	0.0343	2.199	0.2547
Se[a] (95% CI)	98.4 (91.57 – 99.74)	92 (73.93 – 98.78)	92.2 (82.69 – 97.39)
Sp[b] (95% CI)	85.7 (77.53 – 91.77)	92.4 (84.94 – 96.87)	87.6 (79.76 – 93.23)
PLR[c] (95% CI)	6.89 (4.31 – 11.02)	12.09 (5.88 – 24.88)	7.45 (4.45 – 12.45)
NLR[d] (95% CI)	0.02 (0.00 - 0.13)	0.09 (0.02 - 0.33)	0.09 (0.04 - 0.21)

[a] Se: sensitivity; [b] Sp: specificity; [c] PLR: positive likelihood ratio; [d] NLR: negative likelihood ratio

Table 2. Diagnostic performance of multiplex bead assay and conventional ELISA for *B. suis* (whole cell *B .suis* 1330 antigen) in wild boar sera using a validated ELISA as the gold standard

	Multiplex bead assay	Microarray	Conventional ELISA
Sera	29 positive, 39 negative	27 positive, 36 negative	29 positive, 39 negative
Cut-off	0.01858	1.515	0.6423
Se[a] (95% CI)	100 (87.94 - 100)	100 (87.11 - 100)	100 (87.94 - 100)
Sp[b] (95% CI)	97.4 (86.47 - 99.57)	91.7 (77.51 - 98.15)	100 (90.89 - 100)
PLR[c] (95% CI)	39 (5.63 – 269.96)	12 (4.06 – 35.46)	
NLR[d] (95% CI)	0	0	0

[a] Se: sensitivity; [b] Sp: specificity; [c] PLR: positive likelihood ratio; [d] NLR: negative likelihood ratio

Table 3. Diagnostic performance of multiplex bead assay for *T. spiralis* in wild boar sera using a commercial ELISA as the gold standard

	Assay Platform	
	Multiplex bead assay	Microarray
Sera	21 positive, 97 negative	8 positive, 75 negative
Cut-off	0.04783	4.5426
Se[a] (95% CI)	90.5 (69.58 - 98.55)	75 (35.05 - 96.07)
Sp[b] (95% CI)	99 (94.37 - 99.83)	98.7 (92.77 - 99.78)
PLR[c] (95% CI)	87.76 (12.43 - 619.82)	56.25 (7.71 – 410.5)
NLR[d] (95% CI)	0.10 (0.03 - 0.36)	0.25 (0.08 - 0.84)

[a] Se: sensitivity; [b] Sp: specificity; [c] PLR: positive likelihood ratio; [d] NLR: negative likelihood ratio

Table 4. Comparison of the results among multiplex bead assay, microarray and conventional ELISA for *M. bovis* (recombinant MPB83 antigen), *B. suis* (whole cell B. suis 1330 antigen) and *T. spiralis* (E/S T. spiralis antigen) in wild boar sera

Antigen	**Recombinant MPB83**	**Whole cell *B. suis* 1330**	**E/S *T. spiralis***
		P value	
Multiplex bead assay vs microarray	0.063	0.5	0.625
Multiplex bead assay vs "in house" ELISA	0.07	1	
Microarray technology vs "in house" ELISA	0.687	0.250	

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Chapter 5: Final comments and future perspectives

In the first part of this study, we conducted a large expanding serological survey of Greek wild boar (*Sus scrofa*) in order to profile their antibody reactivity against a variety of pathogens with ecomomic and/or zoonotic importance using conventional serological tools, such as ELISA and IFAT. The results highlight the possible role of wild boar in the circulation of viral, bacterial and parasitic pathogens in Greece and point out to the possible role of these species as sentinels for the surveillance and monitoring of emerging and re-emerging diseases in our country. In the future, gathering information about wild boar serological status at regular intervals will permit us to take preventive measures for the protection of wildlife conservation, the outdoor pig breeding farms, the domestic pig farms and the humans who interact with the wildlife, such as veterinarians, hunters and farmers. The use of geographic information system (GIS) contributed to the demonstration of the hotspots of seropositive and seronegative animals, and the mapping of environmental factors associated with low or high seroprevalence. Worth noting that the high seroprevalence rates in wild boar hunted in the borders of our country indicate the possible spread of infectious agents due to wildlife migration across Greek borders. The rapid demographic expansion of wild boar in Greece, the evidence of their exposure to various pathogens (with some of them being of public health importance), the unclear evidence of wild boar's infectious status in neighboring countries and the increasingly outdoor pig breeding, make regular monitoring of wild boar transmissible diseases essential. Additionally, the use of the GIS as an ancillary tool, describing and analyzing diseases events and identifying areas in need of research contributes in planning, controlling and implementing preventive measures. Our study may serve as the beginning of a surveillance program for wild boar diseases at national level and encourages the collaboration of veterinarians and GIS specialists in order to create a database system designed around wild boar populations and environmental disease events.

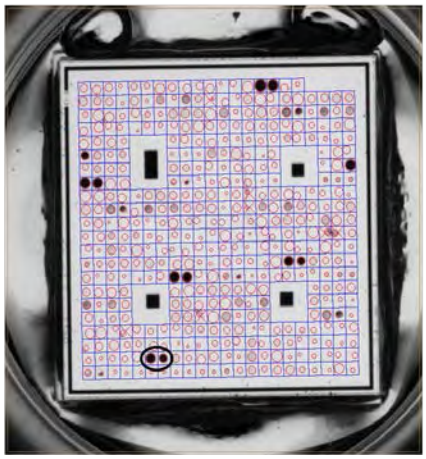
The aim of the second part of this study was the development and evaluation of two novel technologies, antigen microarray and multiplex bead assay, for simultaneous detection of antibodies against *M. bovis*, *B. suis* and *T. spiralis* in wild boar

population, and the development of a multiplex bead assay for the detection of serological responses against *Brucella* species that overcomes the cross-reactivity with *Y. enterocolitica* O:9 in domestic pig and wild boar. Our results indicate that both, the microarray technology and the multiplex bead assay can be used as serodiagnostic tools, as their diagnostic performance is equally good or better than the existing conventional tests. Their main advantage over the conventional tests is their ability to detect antibodies against a range of different antigens at the same time, thus offering significant benefits such as reduced reagent costs and small quantity of serum samples. Another advantage of the specific techniques that have been developed is the use of a non-species specific secondary antibody (protein A/G), permitting the application and evaluation of these multiplexed assays in other wildlife species or domestic animals in the future. These advantages provide a strong impetus for their future use in research and clinical laboratories. Furthermore, the development and validation of these techniques for detection of antibodies against more than three pathogens could become an extremely useful tool for the surveillance and monitoring programs of wildlife diseases, collecting a maximum of diagnostically relevant information about their serological status.

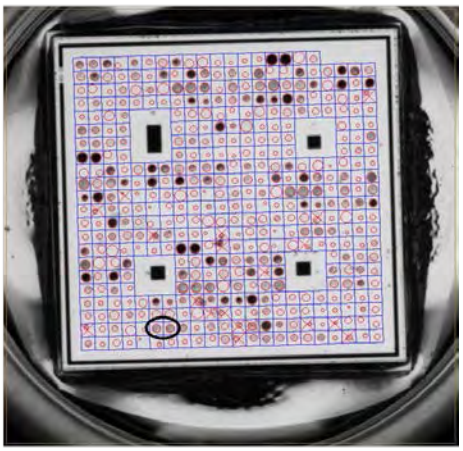
APPENDIX

Fig 1: Examples of positive (black dots) and negative reactions of known infection status serum samples in protein serological microarray

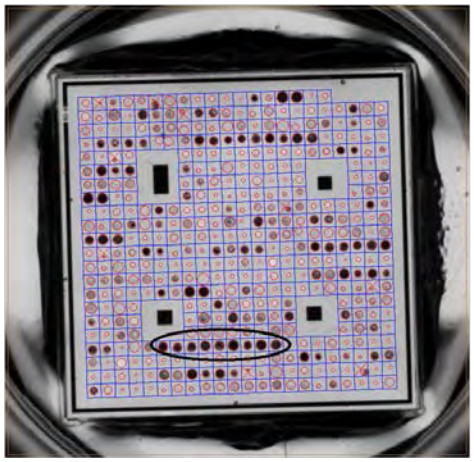
TB positive sample



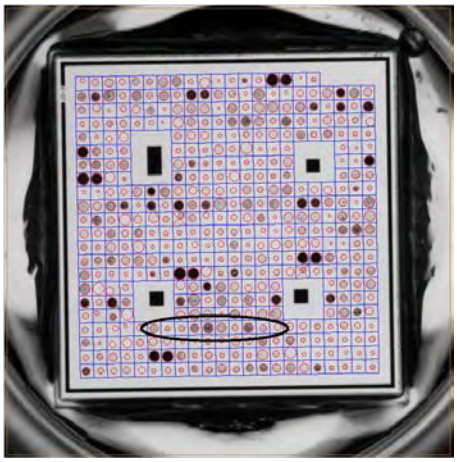
TB negative sample



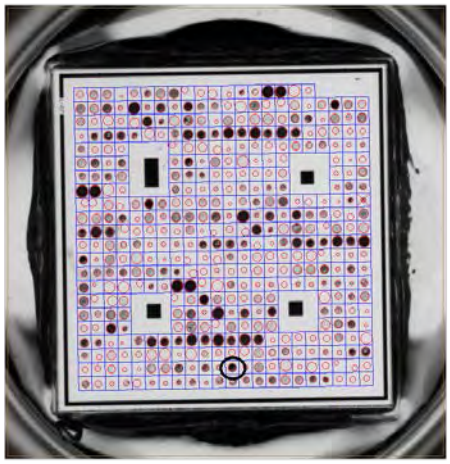
Brucella suis positive sample



Brucella suis negative sample



Trichinella spiralis positive sample



Trichinella spiralis negative sample

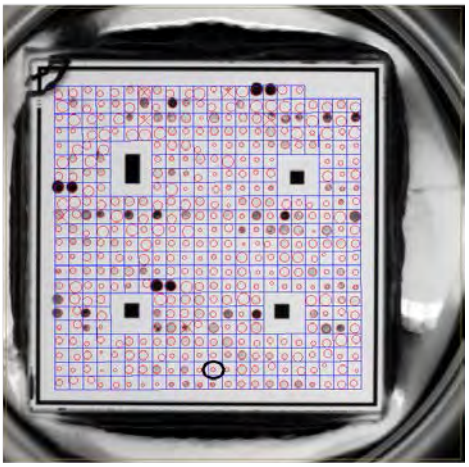


Fig 2: The normalized MFI values for the two *Brucella* spp antigens and the *Y. enterocolitica* O:9 antigen for the *Brucella* infected domestic pigs

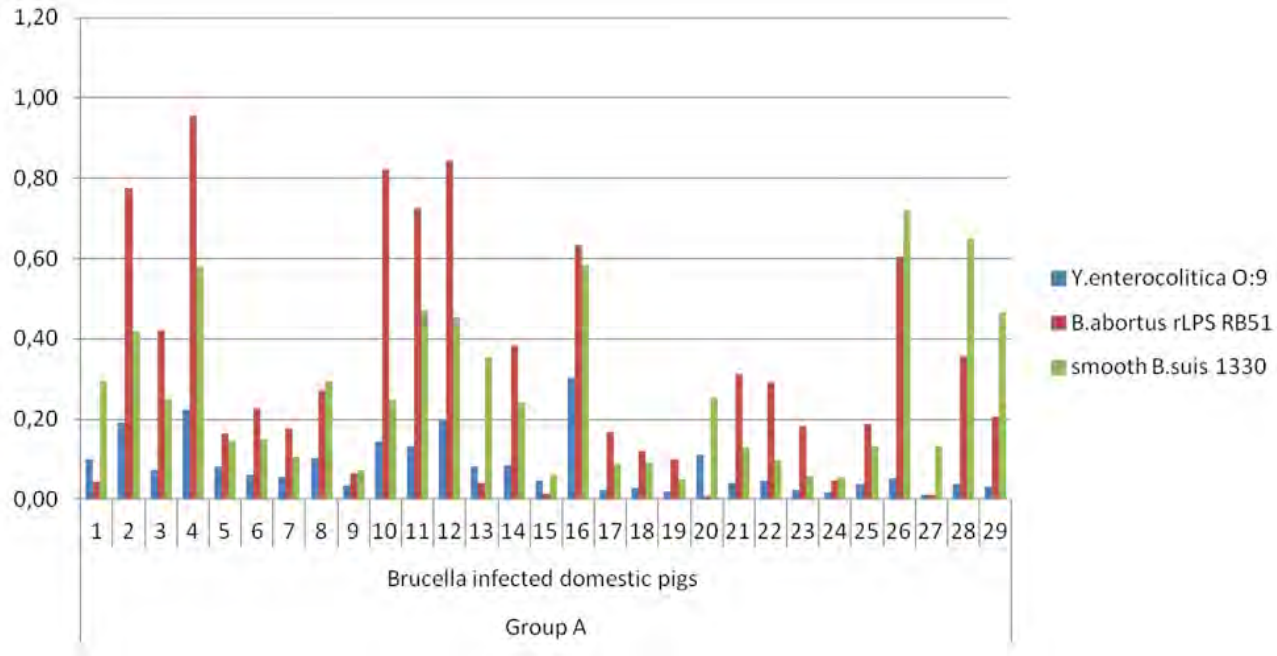


Fig 3: The normalized MFI values for the two *Brucella* spp antigens and the *Y. enterocolitica* O:9 antigen for the non-*Brucella* infected domestic pigs

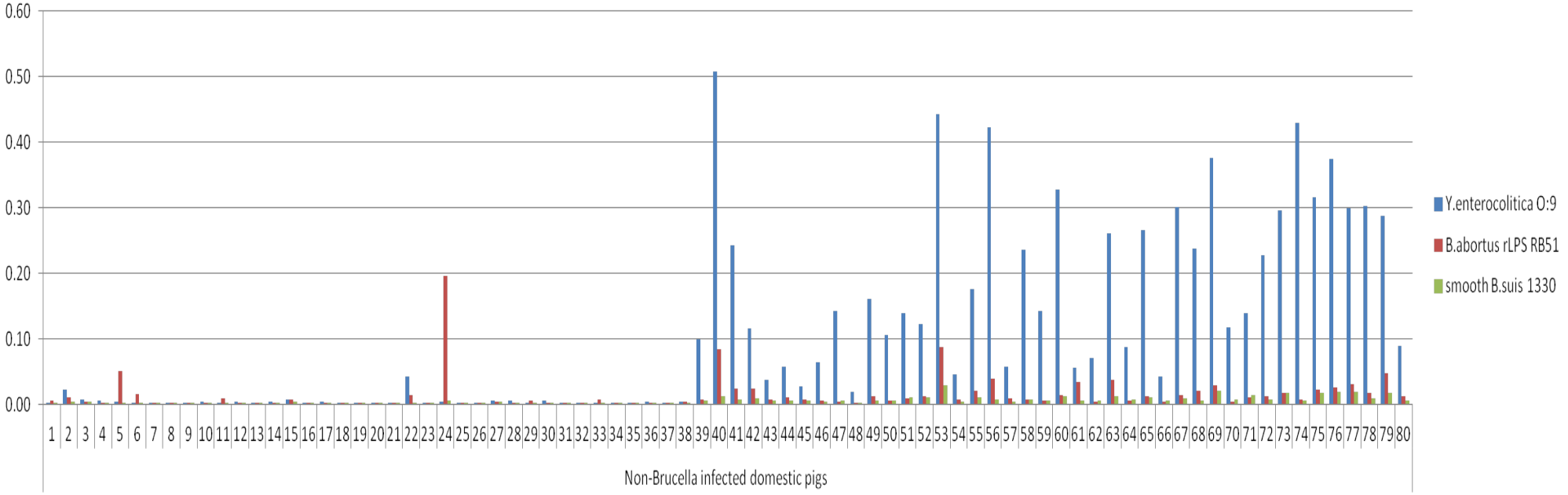


Fig 4: The normalized MFI values for the two *Brucella* spp antigens and the *Y. enterocolitica* O:9 antigen for the *Brucella* false positive serological reactors domestic pigs

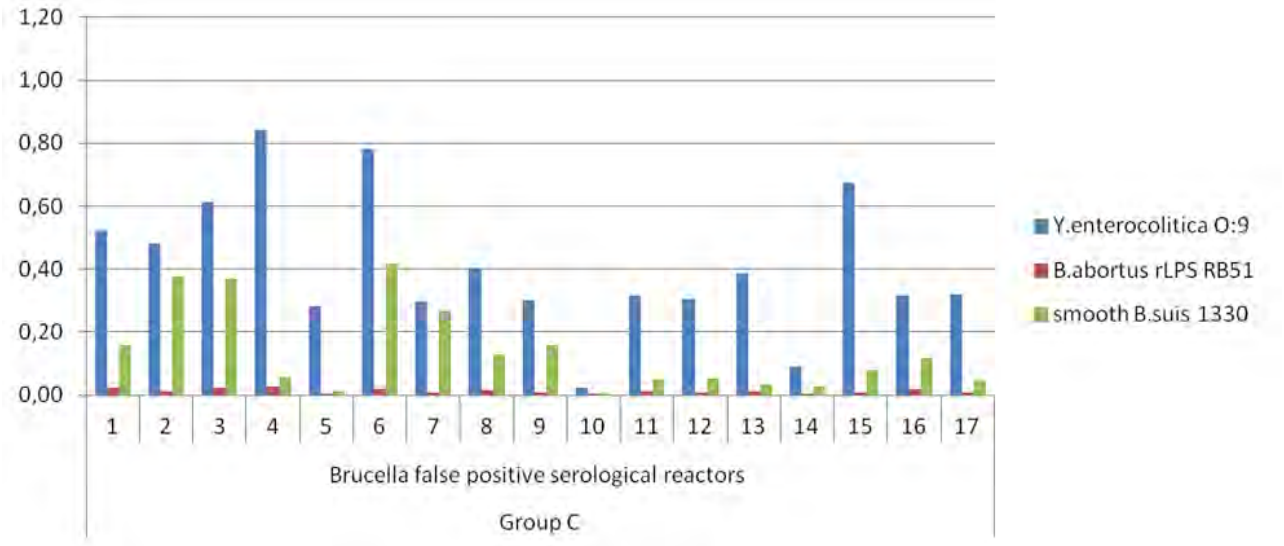


Fig 5: The normalized MFI values for the two *Brucella* spp antigens and the *Y. enterocolitica* O:9 antigen for the *Brucella* seropositive and for the *Brucella* seronegative wild boar